Proteolytic depilation of lambskins

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Abstract

The processing of lambskins plays an important role in the New Zealand meat industry. The use of enzyme dewooling offers considerable advantages over the conventional depilation method which generates unpleasant working conditions and poses product quality risks when not properly handled. Prior to this work it was unclear from the literature why the practice of enzymatic depilation had not generally been adopted by industry. The aim of this work was to determine the problems associated with enzymatic depilation and provide a mechanistic understanding of the dewooling and damaging processes of enzyme depilation to provide underpinning knowledge for the design of a successful enzymatic depilation system.

It was found that variability in depilation between different regions of the skin resulted in either over exposure of the skin to the enzyme regent and subsequent damage or underexposure of the skin to the enzyme reagent and incomplete depilation. Two approaches were taken in the work: Firstly an attempt was made for the first time to understand the variability in enzymatic depilation so that the variability observed in enzymatic depilation could potentially be reduced, thereby allowing a complete depilation process with no overexposure. Secondly an investigation was made for the first time to understand the cause of damage to skins during the process of enzymatic depilation so that the enzyme depilation process could potentially be modified to avoid damage.

Experimental work characterising the time course of depilation and damage development was carried out and compared with the variation of physical properties across the skin. Correlations between depilation and physical properties such as thickness, grease content and follicle density were found. Reduction in the variability of these properties would likely improve the evenness of depilation but would not reduce it enough to eliminate damage due to over exposure.

A range of techniques including: immunohistology, 2-dimensional electrophoresis, matrix assisted laser desorption ionisation, and atomic force microscopy were used to probe the structural and biochemical mechanism of enzyme depilation and damage. In this way it was found that the removal of minor collagen components were the likely cause of damage observed. In particular the removal of collagen VI was associated with a disruption of the smooth mesh of fine collagen fibres observed at the surface of the leather.

The key requirement identified for a successful enzyme depilation system was the use of a broad spectrum protease which has no activity against collagen VI. The means to select a protease with these attributes was also developed by adopting a micro depilation assay incorporating immunohistology. This knowledge will enable the future development of non damaging enzyme depilatory reagents that could revolutionise the industry.

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Success is the ability to go from one failure to another with no loss of enthusiasm

Sir Winston Churchill (1874 – 1965)

If you're not part of the solution you're part of the precipitate

Anon

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Preface: Thesis overview

In order to contribute to the knowledge of enzymatic depilation of ovine pelts the following work is reported:

- Chapter one entails: A definition of the problem at hand, the requirements for work done in this thesis, and describes the approach taken for this work.
- Chapter two contains a detailed description of conventional lime sulfide based depilation and its associated problems; a description of the possible alternatives to lime sulfide based depilation and how they relate to enzyme depilation; a detailed description of the structure of the skin and the bio-chemicals in the skin; and a description of the current understanding of the mechanism of enzymatic depilation.
- Chapter three includes an evaluation of the barriers to a successful enzymatic depilation process and a determination is made of what work was required to understand the enzyme depilation mechanism and so enable the prediction of a successful enzymatic depilation process.
- Chapter four builds on conclusions from chapter three and assesses the issue of variability in depilation across the skin by investigations of heterogeneity in the skin, thickness and diffusion through the skin, and skin composition.
- Chapter five assesses the protein and protease requirements for the production of high quality leather from enzyme depilated material including an assessment of the literature and analysis of protein changes in enzyme depilated skin compared with conventionally depilated skin. Parts of this chapter have been published in (Allsop *et al.*, 2007) and (Edmonds *et al.*, 2008). Immunohistology incorporating cross sections of lambskin exposing all the different layers described in chapter 2 was used extensively in this section
- Chapter six investigates the possibility of controlling the adverse effects noted during enzymatic depilation through the use of salts, and the use of targeted enzyme reagents.
- Chapter seven concludes this thesis, discusses the findings and their implications in enzymatic depilation and suggests possible new directions of research.

Glossary

The following glossary was prepared from a selection of reference sources ((Roether, 1976),(van der Loo *et al.*, 1999), (Harris, 1974))

Table 1: Glossary

word	description
Bate	Reagent applied during bating, usually an enzyme product such as pancreatic trypsin. (n) The process by which interfibrilar proteins are removed and the collagen structure opened up usually carried out after liming at pH 9 and 35°C. (v)
Beamhouse processing	The process of loosening wool or hair from skins and removing non-collagenous proteins from the hide or skin and preserving the skin through a pickling process in such a way that it is ready for tannage. Eg see “conventional processing”
Conventional processing	In the context of this thesis conventional processing is the lime-sulfide based process whereby raw skins are painted with a solution containing hydrated lime, and sodium sulfide, which loosens the wool. The pulled slats are then limed, delimed, bated, and pickled.
Crust	A skin that has been tanned retanned, dyed, and dried but not yet finished.
Delime	The process by which the pH of a slat is brought down from around 12 or 13 down to 8 or 9. This is usually carried out in a processing vessel using a deliming agent such as carbon dioxide or ammonium sulfate.
Depilation	see "Dewool".
Dewool	The process by which skins are treated so as to loosen the wool to an extent that it can be removed easily from the skin.
Drum	A rotating vessel in which skins are processed. (n) Apply mechanical action in a drum through rotation of the vessel. (v)
Enzyme	A protein which acts as a catalyst in chemical reactions especially lysis reactions.
Fell	Describes the skin of an animal as it is removed after slaughter.
Fellmongery	The place at which raw skins are processed to remove wool or hair and other unwanted proteins from a skin as it is converted into a pickle.
Finish	A coating applied to the surface of a finished leather. (n) The process of applying a coating to crust leather in order to generate finished leather. (v)
Finished leather	Leather that is ready for use in a leather object such as a garment or as upholstery for example.
Flesh	The side of the skin that was inside the animal. (n) The process of removing excess fat and muscle from the flesh side of the skin prior to processing. (v)
Float	The liquid in the process vessel in which skins and reagents are drummed. (n) To add liquid to a drum to achieve a desired liquid level. (v)

Grain	The outermost layer of skin remaining after successful processing to crust leather.
Green skin	A skin immediately after its removed from animal at slaughter (also known as raw skin).
Hydro extract	In the context of this work this process involves the use of the LASRA hydro extractor which spins the skins at a constant rate for a constant amount of time and produces a skin with a consistent quantity of associated water.
Liming	The process in which the residual wool and soluble proteins and glycosaminoglycans are removed from the skin after the bulk of loosened wool has been removed.
Opening up	The process by which collagen fibres are separated during processing.
Official sampling position (OSP)	A position on a skin near the back bone and about one third of the way from the butt to the neck. (IUP2, 2000)
Paint	A solution containing depilatory reagents usually applied in a thickened form to the flesh side of a skin. (n) The process of applying a depilatory paint usually to the flesh side of a skin. (v)
Pickle	An intermediate stage in the process of converting raw skins into leather when the skin is preserved in a highly acid state in the presence of salt. (n) The process of salting and acidifying a skin from which the unwanted proteins have be removed. (v)
Potting	The period during which the skins are held after they have been painted with depilatory during which the chemicals penetrate the skin.
Pre-fleshing	See flesh (v).
Protease	An enzyme that catalyses the hydrolysis of proteins.
Pulling	The process by which the wool is removed from the skin after it has been loosened by a depilatory.
Shrinkage	The thermal denaturation of collagen resulting in a change in morphology of the collagen fibres with an associated loss in length.
Slat	The skin after the loosened wool has been removed but before any processing has been carried out.
Slip	Loosened hair or wool.
Wet blue	A skin that has been tanned with chromium but is still wet and has not yet been retanned or dyed.
Wetting back	The process of taking a salted skin or dry pickled pelt and drumming with liquid in order to rehydrate the skin so it can be further processed.

Introduction

1.1 Definition of the problem

Interest in enzyme depilation comes and goes with comprehensive reviews appearing every few decades (Green, 1952), (Yates, 1972), (Brady *et al.*, 1989a), (Cantera, 2001d). Continued interest in the subject (Sivasubramanian *et al.*, 2008) supports the assertion that there is a widespread desire to use enzymes as the means of depilating skins. The desire to use enzymes as an alternative to conventional lime sulfide processing is based on the concept that conventional lime sulfide processing is hazardous and unpleasant. Conventional processing is used to generate high quality leather and alternatives to its use would have to match it or would not be acceptable.

Examples exist in the literature which describe wholly enzyme based depilation processes (Macedo *et al.*, 2005),(Sivasubramanian *et al.*, 2008),(Saravanabhavan *et al.*, 2005),(Rose *et al.*, 2007), that purport to be successful. However, problems have been reported when ostensibly successful methods have been trialled under New Zealand conditions (Lowe, 1999),(Lowe, 2000),(Cooper & Lowe, 1997),(Cooper, 1997), and to date, no enzyme depilation system has achieved a level of performance approaching that of the traditional lime sulfide process. Briefly; the major problems are: incomplete depilation requiring further sulfide based processing; damage to the grain surface resulting in a nubuck effect; and looseness. Looseness is a fault in the leather in which the internal structure appears to lose its elasticity, and manifests itself as surface creasing when the leather is folded inwardly (Coulson, 1969). By examining the mechanism of the enzymatic depilation process and advancing the knowledge in this area it is hoped that the potential and perceived problems associated with the enzyme based depilation of New Zealand lambskins will be identified and their causes elucidated so it can be implemented in New Zealand without delay.

Although enzymes are able to depilate sheep skins (Yates, 1972), the resulting pelts lack the quality of conventionally processed skins and the process is not used commercially. The mechanisms of depilation are partially understood (Cantera, 2001a),(Cantera, 2001b),(Cantera, 2001c),(Cantera, 2001d),(Brady *et al.*, 1989b). However the effects of the enzymes used in depilation processes on the skin structure need to be examined at

the molecular level so that a targeted depilation regime can be developed that does not result in unwanted damage to skins (Wang *et al.*, 2005). Methods need to be developed that will result in a consistent high quality leather. The problem that will be investigated is therefore that there exists a desire to carry out enzymatic depilation industrially but quality problems in the product act as a putative barrier to the uptake of this process.

1.2 Aims and objectives

The overriding aim of this work was to determine the requirements for a successful enzymatic depilation process for lamb skin. In order to achieve this aim the following objectives were proposed:

- Describe the skin structure and composition to fully define the context for judging the effects of different processes on lambskin.
- Assess the major problems with available enzyme depilation technology by comparing skin structure and composition of pelts subjected to conventional and enzymatic depilation regimes
- Determine the requirements for a successful enzymatic depilation process

1.3 Approach

Depilation of skins involves the removal of wool from the skin. Except for physically pulling the wool from the skin, the process involves the selective removal or destruction of material in the skin to ease the release of the wool. There are therefore two possible paths towards a successful process; either to determine what material needs to be removed then to specifically target its removal, or to determine the material that is important for the integrity of the product and to avoid enzymes that might destroy that material. This work will focus on both pathways towards a viable depilation process as it is possible that both aspects will be important in the final process.

Literature review

2.1 Introduction

New Zealand processed over 30 million sheep and lambs in 2001. The pelts from these animals provided $952 million in exports exceeding earnings from wool over the same period (Passman, 2002).

Fellmongering is the process of removing valuable wool from raw (or green) wool skins prior to processing those skins into pickled pelts (Harris, 1974). In the traditional fellmongering operation, skins are removed from the animal, washed, sprayed on the "flesh" side with a sodium sulfide solution, then held for a period of time, after which the wool (now loosened) is pulled from the skin and the resulting "slat" is processed on to a pickled pelt (Daniels, 2002b).

Traditional fellmongering has several disadvantages that are related mainly to the use of sulfide based depilatory chemicals. There is therefore a major incentive to move away from the use of sulfide (Sundar *et al.*, 2006). The major disadvantages of conventional processing are listed in section 2.2.10

2.2 Conventional process of fellmongery

Fellmongering is the process by which skins are removed from the animal and processed through to the pre tanning stage or pickled skin stage. Conventional processing is described below and the "standard" conventional processes used when required in this work is detailed in section 8.1. The different procedures are described in this work to place the concept of enzymatic depilation in the context of the state of the art method for depilation of lambskins.

2.2.1 Flaying

The removal of skin from an animal is carried out at the meat processing works or abattoir (Roether, 1976). The skin is cut down the center of the belly and pulled from

the carcass (Daniels, 2002a). It comes off the animal at 35°C and then typically is put through a cold water wash which reduces its temperature to about 10°C before it is tumble dried in a continuous perforated tumbling device known as a "continuous hydro-extractor" to remove excess water from the raw skins (also known as green skins). For convenience and to enable transport the skins are usually held overnight (16-24 hrs) prior to processing. For longer term storage the skins may be salted after tumble drying, which dehydrates the skin thereby preserving it (O'Flaherty *et al.*, 1956). Salted skins require rehydration and the excess salt to be removed (known as "wetting back") prior to further processing. For the most part, skins are not salted in New Zealand but are processed as fresh green skins (Passman, personal Communication).

2.2.2 Pre-fleshing

Skins arriving at the fellmongery are generally "pre-fleshed" to cut away the connective tissue and fat still attached to the flesh side of the skin leaving a smoother cleaned appearance (Edmonds, 2003b). For this operation the skins are fed with the flesh side up under a roller that has two helical blades interwoven along its length so that the adhering tissue is cut away without damaging the skin. The purpose of pre-fleshing is to: reduce the level of depilatory required; improve the level of wool recovery, reduce reagent usage during pelt processing; and improve pelt presentation (Edmonds, 2003b), (Passman, 1984). Pre-fleshing is not carried out by all processors but if the presence of fat and meat on the flesh side of the skin presents a significant barrier to enzyme depilation it may become a requirement rather than an option.

2.2.3 Painting

After pre-fleshing, skins are spread out on a conveyer belt flesh side up and passed under a set of spray nozzles, then sprayed with a solution of sodium sulfide and lime (Daniels, 2002b). The paint may be thickened with pre-gelled starch products and may contain a proportion of sodium hydroxide (Passman, 1986),(Edmonds, 2002),(Guns *et al.*, 2003). The chemicals penetrate through the skin within 20 minutes (Edmonds, 2007) and begin to dissolve the wool roots, in particular the pre-keratinised zone immediately inside the wool follicle (Addy, 2000). For the wool to be dissolved a concentration of 1.2%(w/v) sodium sulfide (Edmonds, 2001) at a pH of >12.5 (from a saturated lime solution) (Vivian, 1978) is required. The actual pH required for

successful depilation may not be as high as 12.5, but this pH is delivered by hydrated lime and is therefore a convenient level of alkalinity but not necessarily that which is required for depilation. A summary of chemicals used in conventional depilation is given in Table 2.

Table 2: conventional sulfide fellmongering constituents

Ingredient	Purpose
Sodium sulfide	Strong reducing agent dissolves keratin quickly in the presence of alkali
Lime	Provides source of alkali at pH 12.5
Pre-gelled starch product	Thickener (holds paint on skin)
Sodium hydroxide	Source of dissolved alkalinity (added when an increased rate of depilation is required)

2.2.3.1 Mechanism of sulfide depilation

Under the keratin degradation technique of conventional sulfide depilation the cortex of the wool is destroyed. The cuticle collapses and the hair ultimately disintegrates (Addy, 2000). Previous authors used a histological ("SACPIC") stain to evaluate the impact of the conventional alkaline fellmongering process on the wool root and skin (Yates, 1968b). They found that little happened during the first two hours after flesh side application of caustic sulfide solution, which was presumably due to the requirement for the sulfide to penetrate to the wool root. After 6 hours, however, the force required to remove the wool ("depilation load" measured in Newtons per fibre (Maxwell, 1945)) was shown to be significantly reduced. The outer root sheath or ORS (described in detail in section 2.5.4.2.2) was completely destroyed except for a single layer of basal cells. No nuclear staining of the bulb was seen, even though the bulb itself remained largely intact. The inner root sheath or IRS (described in detail in section 2.5.4.2.1) was not affected at this stage and the epidermis was shown to be intact. After 24 hours the reduction in the depilation load was complete (Yates, 1968b). No change was noted in the epidermis under the microscope even though it could easily be removed in sheets, and the wool could be removed with a little force e.g. by an industrial wool pulling machine. Absolutely no nuclear staining was seen in the bulb and the ORS was

completely destroyed. When pulled, the wool fibre broke in the pre-keratinised region leaving nucleated ORS debris in the follicle.

The primary mode of depilation under alkaline reducing conditions is the destruction of disulfide bonds in the pre-keratinised zone of the wool shaft which weakens it sufficiently to break when pulled (Yates, 1968b).

During conventional fellmongery sodium sulfide (Na_2S), a strong reducing agent, is applied to the flesh side of the flayed pelt, (O'Flaherty *et al.*, 1956). Sulfide ions react with water to produce OH^- and SH^- ions. The SH^- ions pass through the skin and dissolve the keratin on the grain side. The reaction is illustrated in Figure 1:

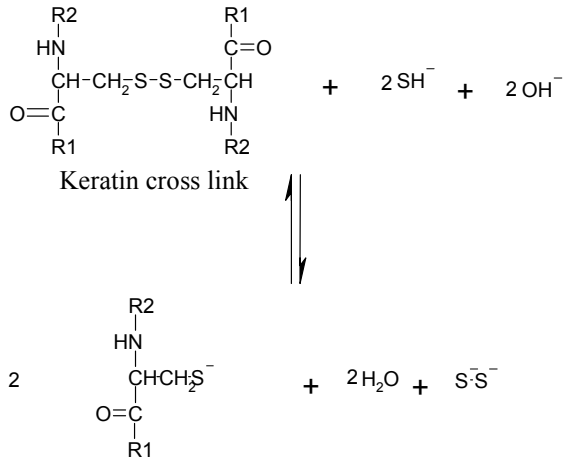


Figure 1: Reduction of keratin by sulfide reaction

The process of keratin destruction has also been described as a nucleophilic substitution reaction (Windus & Showell, 1968). Irrespective of the mechanism, lysis of the S-S cysteine linkage results in breakdown of the keratin structure at the molecular level, which results in a weakening of the wool fibre leading to breakage.

In addition to the destruction of the keratin, however, it is postulated that destruction of the ORS is required as well as the destruction of the pre-keratinised zone of the wool fibre in order to achieve a depilation load low enough for machine pulling (Yates, 1968b). It is interesting to note that once the molecular structure holding epidermis to the dermis had been destroyed, the epidermis could be removed relatively easily as a sheet. However in an investigation of the process of enzyme depilation it was found that the enzymatic destruction of epidermal material did not necessarily correlate with the

depilation (Yates, 1968b). This was probably because hydrolysis of the epidermal material was not carried out under the highly alkaline conditions used in more recent enzyme depilation studies (Passman, 1997). An enzyme depilation process carried out at "neutral" pH was reported in 1968 (Yates, 1968b) while the use of enzymes active at alkaline pH (pH > 8) in the processing of raw skins has been recently described (Passman, 1997), (Cantera, 2001a). If the proteins required to be destroyed for depilation are located adjacent to the epidermal layer then enzymatic destruction of the epidermal layer would not necessarily be expected to correlate with enzyme depilation. It would seem that a level of destruction of proteinaceous material located in the vicinity of the epidermis may be a requirement of any depilation process be it sulfide or enzyme based and that alkaline conditions appear to enhance that process.

2.2.4 Potting (stacking)

After sulfide painting, skins are stacked flesh to flesh and held for a period of time for the paint chemicals to penetrate the skin. If no sodium hydroxide is added the holding time is 24 hours. In the presence of sodium hydroxide the holding time is reduced to 2 to 4 hours (Lowe, 1996).

2.2.5 Pulling

The skins are then passed through a wool pulling machine. The pairs of skins (stuck together flesh to flesh from the paint) are separated and hung flesh side down over wooden boards. The skins (now hanging wool side out) are drawn up between two cylinders spinning in a direction opposite to the movement of the hung skin. The pulling cylinders are similar to blunt versions of the fleshing cylinder. As the cylinders spin they scrape or rub off the wool

2.2.6 Liming

Depilated skins (now called "slats" (Roether, 1976)) are loaded into processing vessels typically in 5 tonne loads. Processing in New Zealand is either carried out in drums or in processing cylinders such as "canbar" processors or "Challenge Cooks". Drums are large even shaped cylinders made of wood or fibreglass with paddle blades attached to internal walls which rotate vertically around the axis to mix the contents. The other style

of processors look like giant cement mixers (Redwood, 1975) and are based on that same design concept adapted for mixing and processing skins (Challenge-Cook Bros, 1977). Water, 60% (based on the weight of skins), is added (called the "float" (Roether, 1976)) and the skins and float are mixed by rotation of the vessel or "drummed". Sulfide from the painting process carries over to the liming process and dissolves the residual wool (Daniels, 2002b). Some sulfide is used up during the potting and liming process. To ensure a consistent result the sulfide levels are monitored after the beginning of liming and are adjusted to 2% (w/v) (Edmonds, 2001). The sodium sulfide, sodium hydroxide, and the lime present buffer the system to about pH 12.5.

In addition to the destruction of keratin, most soluble proteins and proteoglycans are removed from the skin, which results in the collagen fibrils "opening up" (Kronick & Iandola, 1998). This opening up of collagen fibrils is necessary so that the leather produced is not hard or "boardy" (Alexander *et al.*, 1986).

2.2.7 Delime

After the liming material has been washed away, the pH is lowered to between 8-9 using either ammonium salt or carbon dioxide (Lowe, 1997).

2.2.8 Bating

Once the pH has reached an appropriate level a "bate" enzyme, originally a crude pancreatic preparation is added to the skins which are incubated with mixing at 35°C. Bating further opens up the collagen fibrils by removing most of the proteoglycans (Alexander *et al.*, 1986). Some modification of the collagen also occurs. The skin spreads out, in area, and becomes more porous (O'Flaherty *et al.*, 1956). Careful control is required here as over bating will destroy the elastin and result in "loose" leather (Cooper, 1998a). Over bating may also damage the grain surface resulting in dull areas and areas of reduced thickness. Control of these processes is based almost solely on the operator's experience. (In the past operators were known to bite the pelt in order to see if the right kind of impression would be made – astounding when it is considered that bating was originally carried out with dog or bird excrement as the source of proteolytic enzymes) (O'Flaherty *et al.*, 1956). The combined deliming/bating process typically takes 90 minutes.

2.2.9 Pickle

After bating is complete, the pelt is preserved for export/ preparation-for-tannage by pickling. Next 0.9 volumes of water are added (a 90% "float") with 20% common salt. When the salt is dissolved, a solution of sulfuric acid (2% based on pelt weight) diluted 1:10 is added slowly into the vessel. Pickling is carried out over about three hours (Glover *et al.*, 1975).

2.2.10 Problems associated with conventional depilation

The conventional fellmongery process involves the removal of wool from wool skins by exposure of the skins to sodium sulfide solutions in order to remove the hair or wool from hides and skins prior to their conversion into leather This method continues to be used because:

- It is effective
- There is expertise available within industry
- It is cheap/profitable
- It is a robust system offering some level of flexibility
  (Sharphouse, 1971); (Bienkiewicz *et al.*, 1983)

Unfortunately there are a number of negative things associated with sulfide processing:

- The process of sulfide application and depilation is labour intensive.
- The use of sulfide has associated health and safety risks due to the danger of poisonous hydrogen sulfide gas.
- Precise process control is difficult
- Over exposure of the skins to sulfide can cause damage to the skin structure.
- Wool contaminated with the sulfide solution is unusable.
- Sulfide processing results in unpleasant smells and associated air emissions
- Sulfide contaminated effluent is costly to process
- A large number of industrial claims result from damage caused by both alkali and sulfide

(Passman, 1997) (Dempsey, 1976) (Robbins & Manley, 2002) (Thanikaivelan *et al.*, 2005) (Jackways, 1987); (Shivas, 1995) (Mason, 1987)

2.2.11 Conventional processing - Summary

Conventional processing of lambskins to produce pickled pelts entails sequential treatment of the skin with a strong alkaline sulfide solution to loosen wool followed by a prolonged incubation under alkaline conditions (~pH 12.5) which degrades any

remaining wool and removes alkali-soluble proteins (Keller, 1990). The epidermal keratin which is responsible for the hydrophobic surface of the skin is destroyed at this stage of the process (Alexander *et al.*, 1986). After the skin is washed, the pH is adjusted to about 8, the temperature raised to 35˚C, and protease added which breaks-down and removes other non-collagenous proteins from both the interfibrillar matrix of the skin and the upper (grain) surface. This imparts a smooth feel to the surface and improves its appearance (Stirtz, 1982). The skin is then washed repeatedly to remove any residual soluble materials before it is pickled in a salt and sulfuric acid solution at pH 1.0. In this form, it is relatively stable to putrefaction and is ready for tanning. This sequence of processes has a profound effect on the quality of the leather, as it results in the removal of most of the proteins and proteoglycans, except for the collagens and related compounds such as the proteoglycan decorin which is bound to collagen (Kronick & Iandola, 1997); (Kronick *et al.*, 1991); (Deb Choudhury *et al.*, 2006) and elastin (Allsop *et al.*, 2005).

If an alternative method of processing is carried out, then the positive aspects of the conventional process need to be maintained. The alternative process would need to remove all the wool, all the epidermis, the proteoglycans, and leave the proteins that are retained through to the pickled pelt state.

2.3 Enzymatic depilation as an alternative to the conventional process

A promising possible alternative to the use of sulfide is enzyme depilation (Passman, 1997) (Thanikaivelan *et al.*, 2005) (Schraeder *et al.*, 1998). Briefly, enzyme depilation has the following positive aspects:

- The process can be extremely specific depending on the enzyme used
- It would eliminate sulfide contamination of wool
- It would provide a safer and more pleasant work environment
- Eliminates the risk of hydrogen sulfide gas production
- Reduces air emissions
- Provides an effluent nominally free of sulfide

(Passman, 1997), (He *et al.*, 2006), (Thanikaivelan *et al.*, 2000a) (Taylor *et al.*, 1987) (Gehring *et al.*, 2002)

There are a number of reasons why enzyme depilation has not been widely accepted by the fellmongery industry. These include:

- Skin processing at neutral pH typically required by enzymes increases the opportunity for bacterial damage to skins during processing.
- Endogenous proteolytic enzymes that are normally inactive during the conditions of conventional processing may damage the skins during processing
- The need for temperature control as: enzyme reactions are typically orders of magnitude more sensitive to temperature in comparison to physical processes such as diffusion.
- Identification of a depilation enzyme that is effective in the absence of sulfide and produces high quality leather.
- The lack of an enzyme or mix of enzymes that are flexible enough to cope with the inter and intra skin differences encountered at a fellmongery during the year.
- Difficulty in handling enzyme solutions and enzyme exposed skins.
- The inability of an enzyme based process to remove all the wool or hair and so requiring substantial additional sulfide to complete the process. It is clear therefore that this would essentially negate a majority of the benefits of a sulfide free enzyme process.
- Available enzymes that can achieve depilation may also damage the pelt

(Passman, 1997) (Ludvik, 2000) (Tandt *et al.*, 1997) (Germann, 1997) (Felicjaniak, 1985)

Despite over half a century of investigation the exact nature of the causes for the failure of enzymatic depilation are rarely reported. The scarcity of detail of ostensibly unsuccessful enzymatic depilation methods in the literature makes the determination of the exact cause of that failure, difficult. The limited literature (above) detailing failure of enzymatic depilation systems indicates that anecdotally it would appear that damage to the skin and incomplete depilation from a wholly enzyme process are the major reasons for its non-adoption. These aspects of enzymatic depilation are therefore investigated in detail in later sections.

2.4 Other alternatives to sulfide depilation and enzyme depilation

Although sodium sulfide depilation is the current standard for conventional sheep skin depilation a number of other methods have been reported in the literature.

- Hair plucking
- Shearing (band knife)
- High tech laser/water jet shearing

- Cryogenic loosening
- Scalding
- Laser hair disruption
- Electro-depilation
- Oxidative depilation
- Organic acid depilation
- Alternative chemical reducing agents
- Lyotropic agents
- Sheep hormone cycle induction
- Manipulation of known human hair loss mechanisms

The methods are briefly reviewed with their likely application to the modern fellmongery industry and their relationship to enzyme depilation.

2.4.1 Plucking wool

This method of wool removal is the most simple and involves pulling hairs from the skin with no pre-treatment at all. Seemingly straightforward, the problem with plucking hairs from skins is that although a single hair may be removed very simply by this process the force required to remove large quantities of wool from a skin with no loosening pre-treatment results in severe damage to the skin (Gordon, 1980). No technology exists as yet, for the commercial removal of unloosened single fibres.

2.4.2 Shearing

The use of shearing machines based on the band knife shaving technology currently used to split hides and skins has previously been investigated (Gibb, 1987). It was found that any machine would need to leave no more than 1 mm of stubble on the green skin after shearing in order to remove as much of the wool as the conventional lime-sulfide treatment. This is not possible using current band knife technology. No matter how close the “shave” the residual stubble on the skin would remain a problem that could only be rectified by a second sulfide treatment.

2.4.3 Water jet and laser shearing

These techniques for wool removal are new and have not yet been adapted for cutting wool from green skins (Clegg, 1989). Possible problems include potential physical

damage to skin and high developmental and operational costs (Passman, 1997). In addition the problem of residual stubble remains.

2.4.4 Cryogenic depilation

Skins are placed in liquefied gas and cooled to –194°C where the wool becomes loose and can be removed with a comb or brush (Taña & Taña, 1988), (Burton., 1958). Unfortunately fine cracking of the skin surface has been observed possibly due to the mechanical action of removing the wool at this very low temperature where the skin has lost its flexibility (Passman, . The reason the "cryo" process works appears to be because it opens the pores of the skin, exposing the wool shaft(Taña & Taña, 1988). The mechanisms involved in cryogenic depilation have not been investigated, and the method has not been developed, as there have been no new reports since 1988.

2.4.5 Scalding

This method is used for pig skins when the butchered animal is to retain the skin, and for small sheep skin pieces that are processed in a machine, called a slipe master, designed for the purpose (Carrie, 1952). The scalding process involves heating the skin to >55°C. at which temperature the collagen undergoes thermal denaturation or “shrinkage” and the wool root layers split apart thereby loosening the wool (O'Flaherty *et al.*, 1956). This confirms that the destruction or separation of cell layers in the wool root is important for wool loosening. Details of the scalding method in the literature are few as this method cannot be applied to skins which are to be processed into leather because of the serious damage to the skin through thermal shrinkage.

2.4.6 Laser hair disruption

This technique relies on the use of a laser beam with a well defined wavelength to heat the pigments of the hair at the hair root to a temperature that will destroy the cells of both the hair and hair root. The destruction of root cells disables the growth and leaves the skin relatively hair free after a number of sessions. (Stewart, 2003). Unfortunately this method does not result in the loosening of all hairs after one session and requires that the hairs of stubble length. Depilation is effective because of the destruction of the

growth mechanism, but requires multiple sessions. It is not therefore applicable to the removal of wool from "green" skins in an industrial setting.

2.4.7 Electro-depilation

The removal of hairs by passing a current (direct or alternating current) has been described for the removal of hairs in humans (Letchworth & Colton, 1979). In this method single hairs are selected and treated. Work has shown that wool can be removed from green skins using this method but the results are "patchy" (Phillips, personal communication) possibly because the electric current short circuits through some of the fibres missing the follicles. There appears to have been no further research into this method (Phillips, personal communication).

2.4.8 Chaotropic agents

A number of authors have commented on the depilation effects of salts (Burton., 1958), (O'Flaherty *et al.*, 1956), and (Kritzinger, 1948). Exposing skin to chaotropic agents such as solutions of urea or sodium chloride for a period of 3-7 days was found to loosen the hair. This mechanism has been little used, probably because of the length of time required for depilation. It has been postulated that chaotrophs remove soluble substances from the epidermis and hair root thereby loosening the fibre in the follicle (Burton., 1958). It is also possible however, that although such solutions may act as a bacteriostat in the beginning of the process, this may not last, allowing bacteria to grow after a period of a few days resulting in the observed hair loosening. Nonetheless, chaotropic hair loosening may be an important consideration in the enzyme depilation process. The removal of unknown substances by chaotropic agents prior to enzyme exposure may assist the depilation process by improving the access of enzymes to their target sites. This is an aspect investigated in some of the work reported here.

2.4.9 Chemical shearing

The rate of wool growth varies seasonally. Growth involves three phases as follows:

1) Anagen or the phase of increasing growth rate
2) Catagen or the phase of decreasing growth rate
3) Telogen or the resting phase

(Jarratt, 1999)

Some species of sheep naturally shed their wool seasonally during the telogen phase (Blažej *et al.*, 1989). Domestic sheep that have been bred to hold their wool and not shed can be induced to shed by a subcutaneous injection of the protein "Bioclip" (Jarratt, 1999) or by the injection of or ingestion of a chemical agent. (e.g. Cyclophosphamide or mimozone) This will cause the wool to stop growing and produce a weak point that becomes accessible for depilation after 10 to 14 days growth (Cubis, 1977). As the fleece is shed over a few days after injection a net is required to be placed on the sheep to retain the fleece, until it is removed with the net after shedding is complete. There are therefore costs associated with the protein product, nets to hold the wool, and the capital cost of a specialised crush to hold the sheep during the injection, net application and removal processes. Questions also arise about pre-slaughter withholding periods after injection and the length of the new growth of wool on the resulting *post* slaughter (fell) skin. At the point of slaughter it is likely that the skins will have some stubbly wool remaining, which would have to be removed, presumably with a lime sulfide treatment (Cooper & Lowe, 2000). This system of wool removal had therefore not become widely used at the time of writing.

In some respects the "bioclip" process described above mirrors the process of alopecia or "male pattern hair loss". Here, hormones induce the follicle to regress permanently to the resting telogen phase when the hair stops growing (Sawaya, 1991). Both of these effects occur while the animal is alive. In this condition, hair growth reduces during the telogen phase to a point when the fibre becomes so thin that it breaks off. The relevance of this method for inducing hair loss to fellmongery depilation is minimal, as the process is not applicable to the dead skin.

2.4.10 Alternative chemical depilation agents

2.4.10.1 Wholly alkali depilation

Due to the susceptibility of wool to an alkaline environment ((Jackways, 1987)) it has been claimed that it is possible to remove wool commercially with alkali alone in the absence of any sulfide "sharpening" agent (Knaflic & Miller, 1980). This process was designed for hides as a liming process which takes about 12 hours to pulp and remove the hair under mechanical action. It has not become widely used since its inception,

which may be due to the reduced pulping action and slower liming time in comparison to sulfide based liming. No mention of the application of this method to skin depilation with wool recovery has been made. It is possible that the process required mechanical agitation to achieve a dewooled result which would not lead to the method of wool recovery that is desired for lambskins.

2.4.10.2 Oxidative depilation

The procedure for oxidative depilation involves the use of strong oxidising agents such as hydrogen peroxide or sodium chlorite. Oxidative depilation can be done in an acid medium (Chen *et al.*, 2000) or in an alkaline medium ((Marmer & Dudley, 2005b) (Bronco *et al.*, 2005) (Shi. *et al.*, 2003) (Rosenbusch & Kliegl, 1971) (Sundar *et al.*, 2006)). The oxidant reacts with the keratin to produce a soluble keratin sulfonic acid. Raising the pH to 9.5 or greater by addition of NaOH results in the remaining keratin (Rosenbusch & Kliegl, 1971). When calcium is used to supply the alkaline solution "immunisation occurs" and disulfide bonds that have been broken are rendered stable to further sulfide attack by formation of thio-ether bonds under alkaline conditions causing the keratin to remain insoluble (Bronco *et al.*, 2005). It has also been suggested that alkaline solublization of wool is enhanced by the use of oxidative agents which cleave sulfide bridges allowing the fibre to swell and take up more depilatory reagent (Marmer & Dudley, 2006).

This process has not been widely used in New Zealand, most probably because of the high cost of the processing chemicals (Marmer & Dudley, 2005b) compared to sodium sulfide and the hazards associated with the use of strong oxidising agents. Also, the oxidative process modifies the collagen in a different way compared to that of the standard alkaline processing resulting in the solublization of different proteins (Marmer & Dudley, 2007),(Scroggie, 1970). The process does not damage the mature hair shaft as it only dissolves the soft pre-keratin (Bronco *et al.*, 2005). However, after the oxidative treatment some short hairs can remain (Marmer & Dudley, 2005a).

2.4.10.3 Acid depilation

Acid is used in Australia to depilate merino skins (Carrie *et al.*, 1956). The acetic acid used apparently breaks open lysosomes in the skin releasing proteolytic enzymes which in turn loosen the wool. Unfortunately the skins are heavily damaged by this process

when it is carried out at high temperatures such as those experienced in some regions of Australia. However, at cooler temperatures such as those typical of most regions in New Zealand, the process does not adequately depilate skins (Edmonds, 2003a).

2.4.10.4 Other organo-compounds

Organic compounds such as dithionite, thioglycolate and other "mercapto" type compounds are commonly used in personal depilatory products manufactured for the beauty market. These compounds can also be used to remove wool. It has been speculated that these organo-sulfhydride compounds use the same mechanism as sulfide (O'Flaherty *et al.*, 1956). Although the risk of hydrogen sulfide gas emissions is reduced, the sulfur content in the effluent is not, as instead of a sulfide an organo-sulfhydride is released in a 1:1 molar ratio. Added to this, these chemicals are prohibitively expensive in comparison to sodium sulfide.

2.4.11 Alternative depilation methods - Summary

Chemical depilatory agents mostly remove hair by destroying the keratin in the fibre itself, not through attacking the material that cements the fibre into the follicle.
Electro depilation works at the root level but its mechanism is poorly understood. Patent claims revolve around the action of caustic agents released by the electric current into the wool root. It would appear therefore that the mechanism of electro depilation is equivalent to that of chemical depilation.

A number of physical means are available to remove the bulk wool, resulting in a green skin with short stubble. These methods have no direct relevance to enzyme depilation but may serve as pre-treatments to enhance enzyme based skin processing. Understanding of the wool removal pre-treatments for the enzyme depilation is therefore only of partial interest in the context of the present work.

The mechanism of hair loss in a live animal involves the resting of hair growth, which results in thinning, eventually leading to breakage. It is clear that this mechanism of hair loss has no bearing on the removal of hair from a dead skin.

2.5 Skin microstructure

Knowledge of the skin structure of the skin is important for understanding the processing of raw (or green) skin into leather. Certain layers need to be removed whilst the careful maintenance of the integrity of other layers is vital to the production of high quality leather. The skin contains a number of individual layers (as shown in Figure 2) with each containing different biomolecules that tend to be defined differently depending on the focus of the researchers. The overall thickness of sheep skin is about 2.7mm (Kozlowski & Calhoun, 1969).

In order to have a consistent diagrammatic image of skin, the descriptions have been incorporated into a simplified/generalised structure of the skin layers as defined by medical researchers and illustrated in Figure 3 (Briggaman & Wheeler, 1975); (Stanley *et al.*, 1982). The details of the hair structure are discussed later.

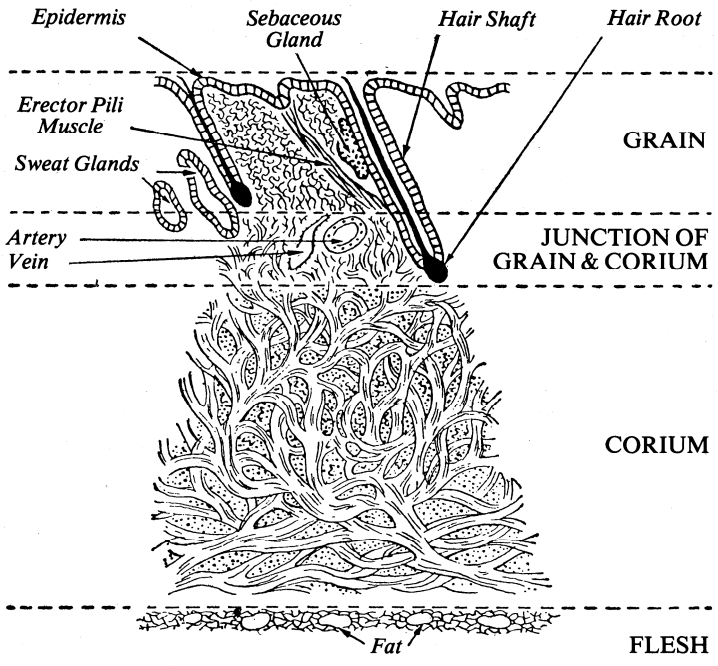


Figure 2: Diagram of skin showing structures important for leather making (Sharphouse, 1971).

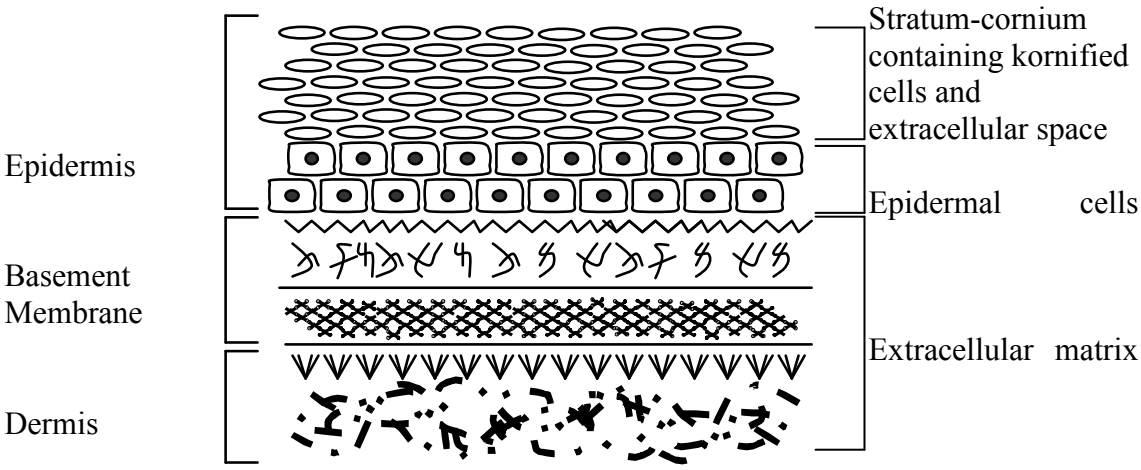


Figure 3: Simple raw skin micro structure medical definitions

It is the dermis, however, that represents the portion of the skin which becomes the final leather product. Although the dermis is often described as a single layer after the diagrams above, names have been attributed for different layers in this material within the field of leather science. These are illustrated in Figure 4 below.

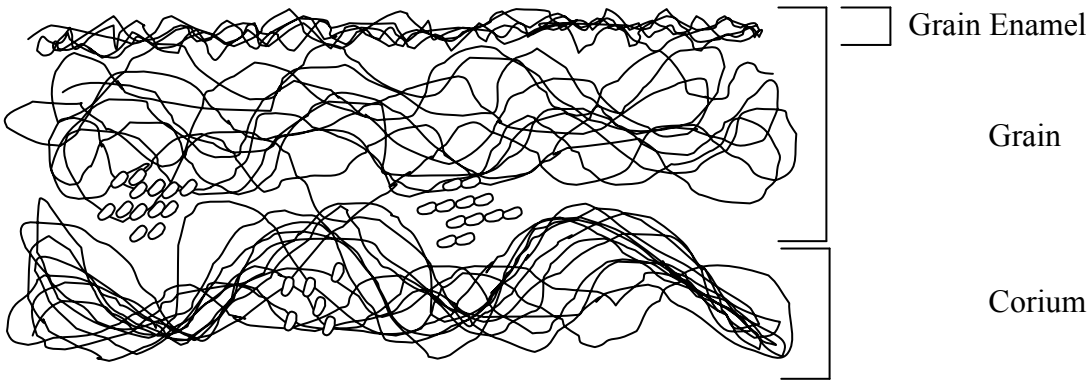


Figure 4: Leather maker's definitions of the different parts of the dermis

The dermis portion of the skin is the part that leather is produced from (Haines, 1984a). When in its final processed form the skin dermis can be further subdivided into the grain and the corium (Haines, 1981). The grain surface has been shown to have a different structure compared to the rest of the grain, and so has been shown separately in the simplified diagram above (Figure 4) and has been designated as the "grain enamel". This part of the skin is known by a variety of names throughout the industry such as the grain surface, fly-wing (when peeling off), and enamel. For the purpose of consistency,

the term “enamel” will be used to distinguish this layer from the rest of the grain for the remainder of this work. The relative thickness of each layer depends on the breed of sheep, but generally the grain enamel is about 100 um thick, with the grain and the corium making up the remainder in approximately even proportions depending on the position across the skin (Dempsey, 1984).The importance of the grain enamel, grain, and corium is discussed in more detail in section 5.5. The most important micro structure of the skin in terms of depilation is, of course, the hair, which is in the dermis. The bulb of which is found near to the junction between the grain and corium as shown in Figure 5 a schematic diagram that is based on photomicrographs of raw sheep skin (Dempsey, 1984).

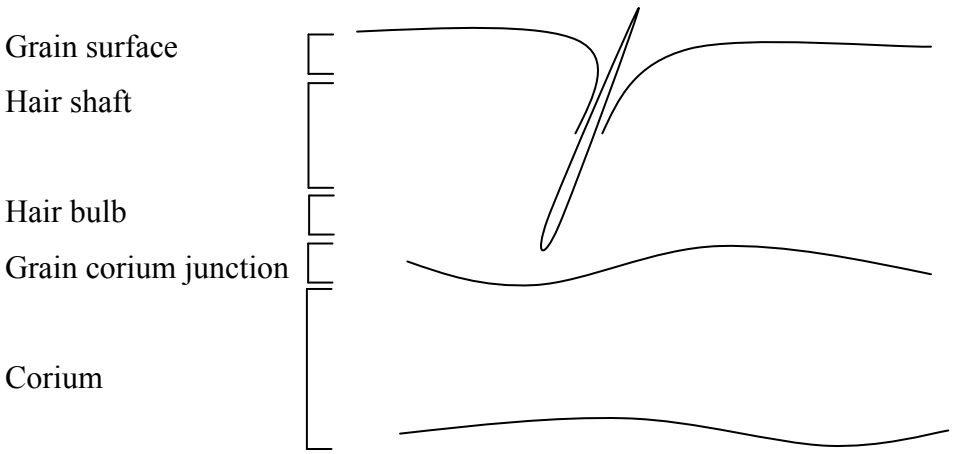


Figure 5: Simple skin macrostructure (from (Dempsey, 1984))

Each of the regions of the skin will be dealt with in turn, starting from the deep extracellular matrix working up to the epidermal cells and then, from the inner part of the hair, working outwards again to the epidermal cells.

2.5.1 Extracellular matrix (ECM)

The extracellular matrix of skin, as its name suggests, is the material found outside and between cells. In the context of this work it specifically refers to the extracellular matrix of the skin which consists of a number of non-cellular proteins that lie outside and beneath the cells of the epidermis as illustrated in Figure 3. The extracellular matrix can be defined as any secreted material that is immobilised outside cells (Reichardt, 1999). The major macromolecular components of the extracellular matrix are: collagens, non-

collagenous glycoproteins, proteoglycans, and other proteins. These are discussed in separate sections.

For the purpose of this review the extracellular matrix can be divided into groups: the basal membrane, the region of cell-cell adhesion which is discussed below, and the collagen matrix discussed in some detail on a collagen by collagen basis in section 2.6.1.

2.5.2 Basal membrane

The basal membrane (basement membrane) is found in skin between the epithelial cells of the epidermis and the extracellular matrix of the dermis (Hay, 1964); (von Loutis *et al.*, 1984). It consists of two regions differentiated by their appearance under the electron microscope. These are; the lamina lucida (or lamina rara) which is relatively electron transparent and the lamina densa which is less so. The two regions are arranged with the lamina lucida adjacent to the cell surface and the lamina densa adjacent to the connective tissue (Charonis & Tsilibary, 1990).

The proteins present in the basal membrane are extensively cross-linked and some are unique to this structure. The major components of the basal membrane are:

Collagen types: IV, VI, VII; laminin; entactin/nidogen; proteoglycans; and fibronectin (Stanley *et al.*, 1982).

Practically all of the macromolecules present in the basal membrane can interact with each other *in vitro*. Although, the exact nature of these interactions are not clear, they generally occur within micro domains and are complicated by the modifying effects of other macromolecules (Charonis & Tsilibary, 1990).

Three models for the structure of the basement membrane have been proposed by Charonis and Tsilibary (1990). These are; the layer model, the matrisome model and the assembly polymorphism model.

i. The layer model describes the basement membrane as layers of collagenous and non-collagenous materials that are sequentially laid down and held in place by both covalent and non-covalent bonds between the macromolecules in adjacent

layers. In this model, the membrane is most likely to be homopolymeric in nature.

ii. The matrisome model describes the basement membrane as an aggregation of stoichiometrically similar building units or matrisomes comprised of standard quantities of basement membrane components. The matrisome model results in heterologous interactions between macromolecules.

iii. The assembly polymorphism model describes the basement membrane structure as a combination of the layer and matrisome models in which the basement membrane is built up by polymers that are assembled as the different macromolecules become available; i.e. regions of both homopolymeric and heterologous interactions can be present (Charonis & Tsilibary, 1990).

A detailed model of the basement membrane has been proposed, (Yurchenco, 1990): Type IV collagens form tetramers which combine to form an irregular three-dimensional network. Laminin polymers also form a network which becomes cross-linked within the collagen network. In this model laminin provides structural support and sites for cell adhesion (Timpl *et al.*, 1990).

If destruction of the basal layer is required for enzyme depilation then it would seen logical to target laminin, fibronectin and collagens IV,VI, and VII.

2.5.3 Epidermis

2.5.3.1 Epidermal structure

The epidermis is the outermost layer of the skin, and is an animal's first barrier to external threats. The epidermis consists of viable cells that grow and reproduce to produce a cornified layer of keratin at the skin surface. During conventional processing the layers of cornified cells are destroyed by the action of alkaline sulfide presumably by the same mechanism that destroys keratin in the wool or hair fibre. The process of removing the epidermis exposes the smooth grain surface, which forms the visible surface of the crust leather, and therefore needs to be completed to form a quality product (Ding *et al.*, 2005).

The efficiency of penetration of "extra-dermal" agents is affected by the keratin of the cornified cells and the lipid filled extracellular space (Al-Amoudi *et al.*, 2005). The extracellular structure has been recently investigated using Cryo-electron microscopy (Al-Amoudi *et al.*, 2005). This work showed that the extracellular space contains multiple lipid bi-layers which probably confer much of the waterproofing to the skin. If a depilatory agent were to be applied from the grain side then it would have to pass either through these bi-layers or down the hair root shaft in order to reach its target.

On the other hand, it has been observed that, strips of cornified material can be peeled from the underlying dermis during enzyme depilation (Yates, 1968b). This indicates that adhesion of the epidermal cells to the underlying extracellular matrix could be important in the enzyme depilation process. The adhesion of epithelial cells in the epidermis is a special case, and two different kinds of adhesion are observed; the adhesion of "like" cells to one another to maintain the cells in a two dimensional sheet and the adhesion of that sheet of cells to the underlying extracellular matrix (Bell, 1988). The epidermis is therefore a structure that acts as a barrier to a grain side applied enzyme depilatory.

2.5.3.2 Epidermal cell adhesion

Cells have a polysaccharide rich coating called the glycocalyx. which is most important in the process of cell adhesion and can be readily stained with ruthenium red (Bell, 1988),(Bongrand, 1988). It consists mainly of proteoglycans although some glycoproteins such as fibronectin are present. The cell surface is not flat but is covered with small protrusions about 100Å apart on average, this distance being somewhere between the length of the glycoprotein side chains and glycoprotein lateral chains (Bongrand, 1988).

Cells attach to each other and the substratum by means of footpads and thin retraction fibres that connect the cell to the foot pads. After treatment with a proteolytic agent the thin retraction fibres are cleaved leaving the footpads behind (Rollins *et al.*, 1982). The footpad material left behind is known as substratum attached material (SAM), and is made up of fibronectin as a major component, the protein actin, which confers to the cell the ability to move over a surface, and various glycosaminoglycans (GAGs)

(Rollins *et al.*, 1982). In particular SAM contains the GAG's chondroitin-4-sulfate and un-sulfated chondroitin and little dermatan sulfate (Rollins *et al.*, 1982).

The adhesion of cells is essentially regulated by receptor ligand interactions (Bell, 1988). For example, one of the biological activities of laminin is to provide a site for binding of cellular receptors (Timpl *et al.*, 1990). The strength of these interactions, which involve multiple non-covalent bonds between specific proteins and polysaccharides, is influenced by pH. Low pH can disrupt the interactions and release the adhesion (Bell, 1988). As both receptors and ligands lie within the glycocalyx of different cells, in order for adhesion to occur the glycocalyx of each cell will have to contact the other and may become compressed in the process. The strength of binding is thus a balance between the repulsive force of the negatively charged oligosaccharides present in the glycocalyx of each cell, and the binding strength between the receptors and ligands(Bell, 1988). The protein epitopes for adhesion interactions can vary between 3 to 10 amino acids in length (Reichardt, 1999). In particular the sequence arginine-glycine-aspartate (RGD) (also found is arginine-glycine-aspartate-serine RGDS) is important, as it functions as a cell attachment site for many extracellular matrix proteins (Reichardt, 1999). If the abolition of these adhesive forces alone was sufficient to achieve wool loosening, then perhaps specifically targeting this sequence with a highly specific protease could achieve depilation with little other damage.

Fibroblasts bind to fibronectin through a complex of polysaccharides that bind to a specific tri-peptide on fibronectin (Bell, 1988). Receptors for laminin are also found on cells that interact with the basal lamina. It is likely that many macromolecules of the extracellular matrix have specific receptors on the cells with which they interact (Bell, 1988). The exact nature of all the possible interactions between cells is complicated and include interactions with a range of proteins such as: integrins, CD44, and syndecan. These proteins (and others) are known to interact specifically with extracellular material and may well be involved in the cell-extracellular matrix adhesion interaction (Reichardt, 1999).

Overall the impression is that many and varied specific interactions are involved in cell adhesion. Of special interest is the difference in interaction between cell-cell adhesion and cell-matrix adhesion of epithelial cells. Flattened cells attached to the substratum

have many attachment sites, some of which, especially those near the edge of the cell are disrupted on exposure to proteases. When this occurs the cells become more rounded and susceptible to shear force and detachment (Rollins *et al.*, 1982). When "Dispase" (a specific commercial proteolytic enzyme with broad proteolytic activity against ECM proteins) is used, cell-matrix interactions are specifically disrupted resulting in the release of the cells as a sheet (Paul *et al.*, 2001). Clearly, therefore, targeting the specific adhesion interactions of interest could result in the release of epidermal cells without compromising other proteins in the skin.

Heparan sulfate cannot be removed from SAM by exposure to the enzymes trypsin, and testicular hyaluronidase or the chaotroph guanidine hydrochloride. In contrast, hyaluronic acid and chondroitin sulfates can be removed by hyaluronidase and guanidine hydrochloride. Interestingly, removal of these components by enzymes does not appear to affect cell adhesion (Rollins *et al.*, 1982). It can therefore be inferred that GAGs do not affect the strength of adhesion. Presumably the proteins within the GAG matrix are responsible.

The adhesion of cells to the substratum appears to occur by a different mechanism compared to cell-cell adhesion (Roberts & Brunt, 1985). A possible model for the cell substratum adhesion has been proposed where heparan sulfate initially binds to “cold insoluble globulin” already present in the substratum before aggregating into large complexes. Concomitantly, fibronectins bind to the developing interaction in a cooperative manner (Rollins *et al.*, 1982). As these cell adhesion interactions age the quantity of heparan sulfate in the adhesive material decreases (Rollins *et al.*, 1982).

To summarise, it appears that although GAGs are involved in causing cell adhesion, fibronectin seems to be the major structural component of the “adhesive”. An enzyme depilatory which hydrolyses fibronectin would therefore be a possible target for inclusion in an enzyme depilatory reagent mix. However, since many proteins are involved in the interactions between the epidermal cells and the extracellular matrix multiple proteolytic specificities are likely to be required in order to achieve complete separation of the cells and extracellular matrix.

2.5.4 Hair structure

One of the primary functions of fellmongering is the removal of wool. Knowledge of the structure of the wool fibre and wool root is therefore important. A growing wool follicle can be divided into two zones. Firstly the transient zone which involves the growth of connective tissue and fibroblasts, and secondly the permanent zone which contains the mature hair shaft that extends out of the follicle mouth, the erector pili muscle, and the sebaceous gland (Montagna & Parakkal, 1974). The transient zone consists of the matrix, cell growth and differentiation, and keratogenous zones. The hair starts at the matrix where a mass of cells begin growth that then differentiates into the different cell types of the different layers of the hair (Straile, 1965).

2.5.4.1 Hair shaft structure

Like a spring onion (or scallion) a hair has annular layers extending from a common base. Moving from the centre of the generic hair outwards the layers are called medulla, cortex, and cuticle. Surrounding the hair's outer cuticle layer inside the follicle mouth are further layers or root sheaths. The next layer (still moving from inside out) is the inner root sheath consisting of Huxley's layer and Henle's layer. Next is the companion layer. Finally is the outer root sheath (Cantera, 2001a).

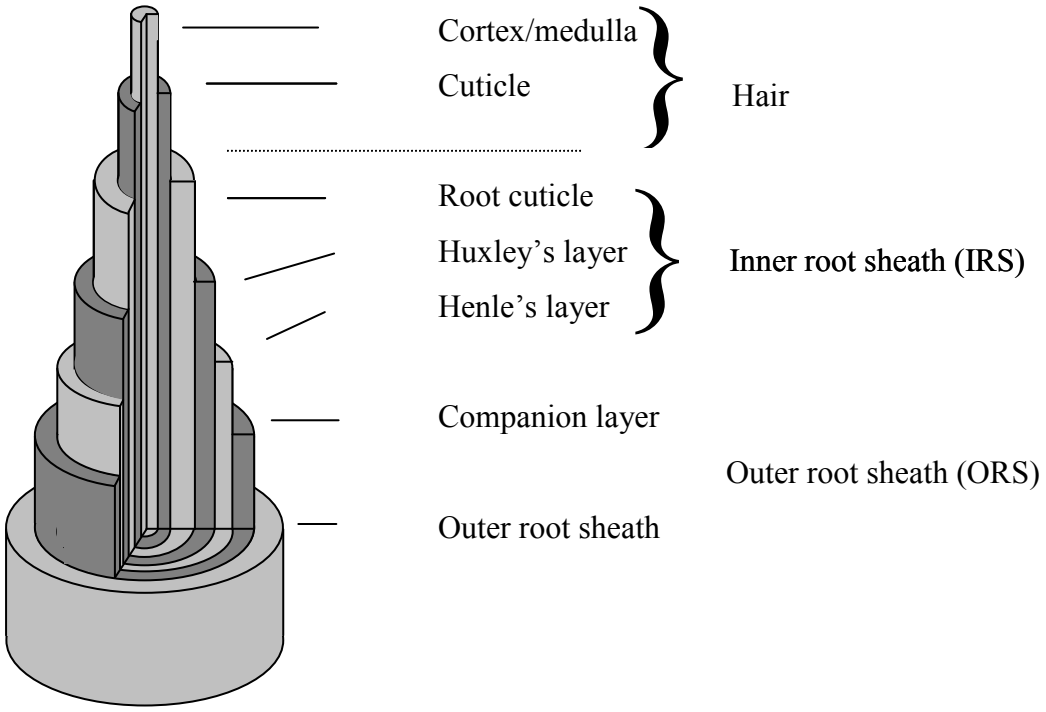


Figure 6: Hair structure (coloured for sake of visualisation)

2.5.4.1.1 The medulla

The medulla is present in the wool of only some of the sheep breeds in New Zealand and then only in an intermediary form in a small portion of some of the wool fibres (Maddocks & Jackson, 1988). The quantity of medullated hairs and the size of the medulla depend on the sheep breed. Specifically the proportion of medullated fibres depends on a range of environmental factors including breed, diet, and the age of the animal. The portion of medullated fibres in the wool from the New Zealand Romney ranges between 1 and 5 % (Scobie *et al.*, 1998).

When present, the medulla exists at the centre of the wool shaft. In merino sheep for example, the medulla consists of an unbroken column along the axis of the shaft that is symmetrically shaped in cross section with a fibrous amorphous infill material. An inner framework supports a shell surrounding the air spaces (Blažej *et al.*, 1989).

2.5.4.1.2 The cortex

The cortex makes up the majority of the fibre. The wool cortex can be differentiated into two parts; the orthocortex and the paracortex. The two types can be observed by differential staining. The cortical cells are about 95 um long and 5 um in diameter and contain a small portion of cytoplasmic debris. Cortical cells consist mostly of keratin that is organised in a structural hierarchy Keratin molecules are packed into microfibrils, which are in turn packed into macrofibrils that make up the bulk of the cortical cells (Maclaren & Milligan, 1981). Under transmission electron microscopy the intercellular spaces appear as a region of protein deposited during keratinisation that is sandwiched between two layers of covalently bound lipids. The central protein layer is known as the δ-layer, and the intermediary lipid layer as the β-layer (Negri *et al.*, 1996). It is quite possible that this material is degraded by so called “keratinases” resulting in the release of cortical cells.

2.5.4.1.3 The cuticle

Surrounding and encapsulating the cortex is the cuticle. The cuticle is made up of scale like cuticle cells which act as a protective barrier. The cuticle cells have two parts; the exo-cuticle and the endo-cuticle and is surrounded by a hydrophobic membrane complex containing covalently bound fatty acids that cements the cuticle together. These lipids differ from those present in the cortical cell membrane matrix in that they

can be removed using methanolic KOH unlike those of the cortical membrane. (Negri *et al.*, 1996).

2.5.4.2 Hair root structure

2.5.4.2.1 Inner root sheath (IRS)

The inner root sheath consists of three layers; firstly the root cuticle that is in direct contact with the hair cuticle, and is followed by Huxley's layer and then by Henle's layer as illustrated in Figure 6.

The root cuticle consists of a single layer of cells that are partially nested into the scale formation of the hair cuticle cells, and helps to hold the hair in place. This layer may also be involved in the formation of the shape of the hair cuticle. Huxley's layer may be more than one cell thick and differences in thickness appear to be associated with changes in the hair diameter. Huxley's layer may also be involved in the transport of nutrients to the hair cells (Straile, 1965).

Henle's layer lies between Huxley's layer and the companion layer to the outer root sheath. It may be involved in the movement of the hair up through the hair pocket and may be involved with nutrient transport to the hair.

2.5.4.2.2 Outer root sheath (ORS)

The outer root sheath is continuous with the epidermis being one to two cells thick around the bulb. The cellular structure is therefore very similar to that of the epidermis.

2.5.4.2.3 Companion layer

The cell layer closest to the inner root sheath becomes differentiated from the rest of the outer root sheath after four or five cells distant from the bulb. These cells become flattened and are known as the companion layer (Orwin, 1979). It is likely that the cells of this layer are involved in the movement of the inner root sheath up the hair pocket and the eventual destruction of the inner root sheath.

2.5.4.3 Hair growth

Cell growth and differentiation begins in the bulb, at the centre of which is the dermal papilla, which supplies nutrients to the growing hair cells. The papilla controls the type

of hair produced. For example a small papilla results in a small hair. Surrounding the dermal papilla is a region of mitotically active cells known as the matrix where cell growth begins. As cells are pushed up past the dermal papilla cell differentiation begins. During growth and differentiation of the cortical cells the intercellular spaces decrease, cell membranes increase in size and the laminar structure of the plasma membrane becomes indistinguishable until it can no longer be detected. Surface glycoproteins which are detectable in the lowest zone disappear as the cells adopt their final shapes and are undetectable in the mature hair. Cell adhesion appears to be required only at early stages of differentiation and is not needed in the mature cells (Orwin, 1979). Specifically targeting this cell adhesion may help to weaken the base of the fibre and assist in depilation processes without damaging the mature wool fibre product.

2.5.4.3.1 Keratinisation

Prekeratin is formed in the differentiating cells of keratinising tissues (Matoltsy, 1965). Prekeratin can be extracted from prekeratinaceous tissue by 6M urea solution which partially breaks down the prekeratin units. Sulfur is present in the keratin extracts in the S-H form. The molecular weight of pre-keratin is 60 – 100 kDa (Matoltsy, 1965) and its amino acid content has been reported by Matoltsy et al. (1964) pg 302. Prekeratin is formed into microfilaments that are about 60-80 Å thick made up from molecules that are around 1050Å long.

As the cells move further up the hair pocket keratinisation begins with cells of the inner root sheath becoming keratinised first. The process begins in Henle's layer followed by the root cuticle followed by Huxley's layer (Orwin, 1979). Keratinisation begins as filaments form in the cell cytoplasm with associated trichohyalin granules. As cells move further up the hair pocket the filaments begin to fill the cell cytoplasm and other cellular components are destroyed or eliminated in order to make room for these growing microfibrils (Montagna & Parakkal, 1974). It has been suggested that the organelles in the inner root sheath (IRS) become incorporated into the hardened IRS (Orwin, 1979). As cells mature, keratin in the macrofibrils acquires disulfide cross links between cysteine groups. Within the cortex, the macromolecular form of the keratin can vary according to the cell type. Macrofibrils are discrete and circular in the orthocortex, but form an amorphous mass in the para-cortical cells (Orwin, 1979). The final

macrofibrilar structure is made up from a number of microfibrils within a sulfur-rich matrix forming macrofibrils.

As the inner root sheath reaches the top of the hair pocket it becomes corrugated, and protrudes from Huxley's layer into the ORS, the protrusions increasing in number as the cells move up until the cells finally compact, break up and are eliminated inside the pilosebaceous canal (Maclaren & Milligan, 1981).

2.5.4.4 Connective tissue sheath (follicular basement membrane)

Surrounding the hair follicle is the basement membrane and surrounding this is the connective tissue sheath. The basement membrane contains mostly sulfated glycosaminoglycans, while the connective tissue sheath is composed of collagen fibres arranged in two layers. The inner layer of collagen fibres lies parallel to the hair shaft whereas the outer layer has fibres lying at right angles to the hair shaft (Orwin, 1979). The connective tissue sheath and the dermal papilla are continuous with each other and share some traits (Couchman *et al.*, 1991). Basement membrane components are found throughout the papilla matrix; for example an observable basement membrane was found between the hair follicle epithelium and the dermal papilla (Couchman *et al.*, 1991).

2.5.5 Skin microstructure - Summary

The skin can be thought of as a number of connected layers: The dermis consisting of predominantly types I, III, and V collagen fibres with small quantities of minor component collagens that are embedded in a mass of proteoglycans. Next is the basal membrane which holds the skin cells to the dermis and is made up of a number of adhesion proteins and collagens. Adhering to this layer are the epidermal skin cells which become more keratinised and cornified as they move towards the surface.

For the purpose of depilation, sheep skin can be described more simply as a number of wool fibres composed predominantly of enzyme resistant keratin surrounded by a number of cellular sheaths embedded in the skin. Hence by enzymatically targeting any one or all of these layers the hair could be removed intact leaving the underlying collagen matrix undamaged.

The aim of fellmongery is the removal of non-collagenous material, keratin (or wool) in particular from the skin. It is clear that this can be achieved by a number of different methods involving destruction of keratin protein in the root, destruction of the extracellular matrix, and destruction or detachment of the cellular sheaths supporting the wool root. Examples of these three methods are conventional sulfide fellmongery, acid hydrolysis, and enzyme depilation respectively.

Conventional sulfide processing involves chemical reduction of the keratin protein. In particular disulfide bridges are broken and the keratin is dissolved. It would also be expected that disulfide bridges are also broken throughout the extracellular matrix denaturing some adhesion proteins.

Acid hydrolysis results in dissolution of the collagen and extracellular matrix cement and leaves the wool relatively undamaged. This method of wool removal can be useful for removing valuable wool from relatively less valuable skin pieces.

Finally, the specific destruction of structural proteins and cellular sheaths around the wool root by enzymes would be likely to loosen the wool. Of greatest importance for enzyme depilation are the myriad of proteins involved in holding the epidermal skin cells to the dermis and the hair roots in place. While general proteolytic attack can destroy enough of the adhesion to enable the removal of hair, it often results in damage to the skin, making it unsuitable for tanning. It may however be possible to target the major cell adhesion proteins more specifically so that the structural collagens are not damaged. Achieving this may also require the breakdown of proteoglycans which provide a physical barrier to enzyme attack.

A list of possible candidates for selective proteolytic attack is given below:
Hyaluronic acid, and dermatan sulfate: A possible barrier to enzyme penetration,
Collagen types: IV, VI, VII, XII: Structural proteins of the basal membrane. These collagens have non-helical domains which are more susceptible to enzyme attack but do not make up the bulk of the valuable structure associated with the grain surface.
Laminin (assists in cell adhesion)
Entactin (assists in cell adhesion)
Fibronectin (a major component of cell adhesion)

Peptides containing the sequences arginine-glycine-aspartate (RGD) or arginine-glycine-aspartate-serine (RGDS). (peptide components common to cell adhesion)

2.6 Skin Components

Understanding the enzyme depilation process requires knowledge of the structure of the skin and the components that may be affected. The following section reviews all the protein components in skin that may be relevant to the depilation process. The relative composition of the main components of hide has been described (Sharphouse, 1971):

Table 3: Composition of hide and skin

Component	Composition of hide (Sharphouse, 1971)	Composition of lambskin (based on hide adjusted for fat)
Water	64%	53.3%
Fats	2%	18.3%
Mineral salts	0.5%	0.42%
Other substances (pigments etc.)	0.5%	0.42%
Proteins:		
Collagen	29%	24.2%
Keratin (hair)	2%	1.7%
Albumins/globulins	1%	0.8%
Mucins/mucoids – glycoproteins/proteoglycans	0.7%	0.58%
Elastin	0.3%	0.25%

All skins are made up of these components with slightly different relative amounts (Sharphouse, 1971). The major differences between lambskin and bovine hide composition are the presence of wool instead of hair, and a higher proportion of fat (approximately 20%) on the basis of dry matter (Waters & Price, 1989).

2.6.1 Collagen

The word collagen literally means glue making. Since ancient times, hides and skins have been processed into leather and gelatine, gelatines (including gelatine glue) being a product of degraded collagen. Collagen makes up 60-80% of the dry matter in skin

(Bienkiewicz, 1983). It is a complex molecule made up from a number of different polypeptide chains each with different biochemical properties. More than 27 different distinct human collagens have been identified (Veit *et al.*, 2006b), some of which are only found in trace amounts (Olsen & Ninomiya, 1999). A collagen protein characteristically has a large rod-like region containing three polypeptide chains. "Each polypeptide forms a left handed helix and assembles with the other two in a right handed super helix" (Ricard-Blum *et al.*, 2000).

2.6.1.1 The collagen triple helix

Each of the three polypeptide chains making up the triple helix contains the repeating amino acid pattern $(gly\text{-}X\text{-}Y)_{100\text{-}400}$ where X is often proline and Y is sometimes hydroxyproline (Bailey, 1991). Typically hydroxyproline represents about 10% of the amino acid residues present in collagen (Bienkiewicz, 1983). The triple helix is stabilised by intermolecular hydrogen bonds between the main chain atoms of adjacent glycine and proline residues resulting in a rigid rod (Bailey & Paul, 1997).

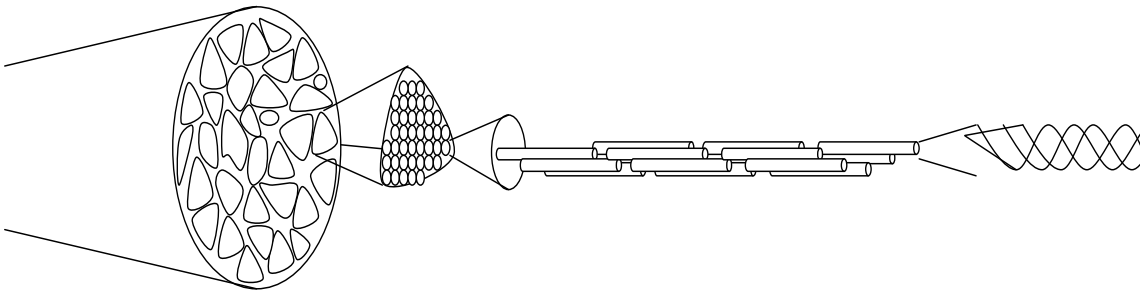
Different collagens are typed according to the α chains present in the triple helix. Some collagens are hetero-trimers containing a mix of different polypeptides. Other collagens are homo-trimers containing three of the same polypeptide such as collagen type II, which contains three α1(II) chains (Kucharz, 1992). Although some types are not found in the skin most are mentioned below for completeness.

The different types of collagen interact with each other in distinct ways and are characterised into four groups based on their macromolecular form (Olsen & Ninomiya, 1999).

2.6.1.2 Fibre forming collagens

Fibre forming collagens include Types I, II, III, V, and XI. Each of the fibre forming collagens forms an aggregate of collagen molecules in a staggered arrangement. Cells excrete pro-collagen into the extracellular matrix. A portion of each pro-collagen molecule is then removed by proteolysis and the collagen molecules aggregate to form protofibrils with a quarter stagger arrangement (Olsen & Ninomiya, 1999) as shown in Figure 7. Each molecule is about 3000Å long and lines up end to end with a short gap between each terminus. Molecules arrange themselves in rafts with one quarter offset at

each end. As this staggered pattern is built up into fibrils a distinctive banding pattern is observed indicating the end-gap and overlap regions (Brodsky & Eikenberry, 1982). The quarter staggered arrangement of molecules within the collagen fibril is visible under magnification as a series of bands that occur every 67 nm (Kühn, 1987). Fibrils in the skin are found together in fibres which are grouped into fibre bundles as shown in Figure 7



	Fibre	Fibril	protofibrils showing stagger	Collagen molecule
Diameter	20-200 um	10-100 nm	1.5nm	(350 kDa)

Figure 7: Schematic magnification of collagen fibre

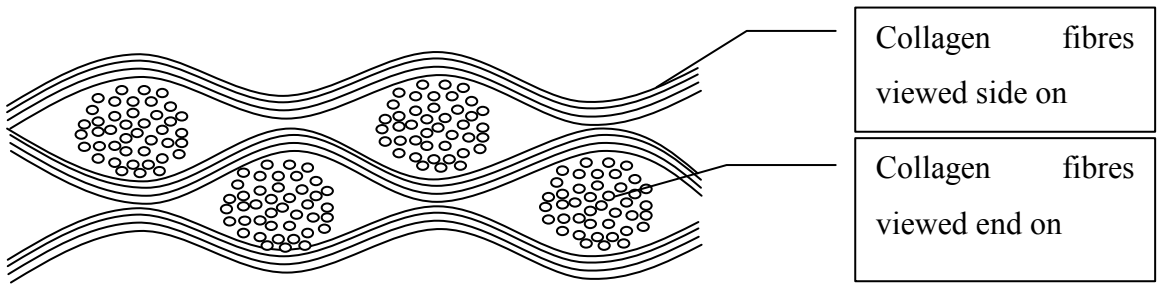


Figure 8: Schematic of collagen weave cross-section (in general the fibres lie parallel to skin surface)

Collagen type I is the most abundant protein in skin. Its main function *in vivo* is to provide a barrier between the organism and the outside world. Type I collagen is very stable to proteolytic attack (Kemp, 1983) and forms a complex weave like structure as illustrated in Figure 8

Collagen type II is found predominantly in cartilage, but not in the skin (Kucharz, 1992).

Collagen type III forms fine fibrils (also known as reticulin fibrils) (Kucharz, 1992), that are usually found within large fibre bundles primarily made up from collagen type I (Kemp, 1983). They are located in the sheath surrounding them and account for 10-20% of the collagen in skin. Because of their location at the surface of the large fibre bundles they are important players in the fellmongering process when the fibre bundles are "opened/split up" (Bailey & Paul, 1997). In contrast to type I collagen, Type III can be damaged by non collagenous proteases (Kemp, 1983). Thus depilation agents are therefore more likely to have a damaging effect on collagen type III resulting in inferior pelts with significant damage to the grain surface where these type III fibrils are located.

Using immunochemistry (including immunofluorometry and immunoelectron microscopy) it was shown that both types I and III collagen are present in thinner collagen fibrils whilst thicker fibrils contain mainly collagen type I with a high concentration of type III collagen at the fibril surface (Fleischmajer *et al.*, 1990). Collagen types I, II, and or V can all be found together in heterotypic fibrils and it has been proposed that fibril diameters are partially controlled by the proportion of type V relative to type I collagen present in the fibril (Olsen & Ninomiya, 1999).

The fibrous collagens are of interest because they are present in the layer of skin that eventually becomes the surface in the finished leather (grain enamel). It could be argued that the collagen structure of this region is the most valuable of all collagen in the skin. The preservation of this layer from uncontrolled enzyme attack and the correct modification through tannage are therefore important in the production of good quality leather (Kemp, 1983).

2.6.1.3 Fibril associated collagen with interrupted triple helixes (FACIT)

Fibril associated collagens include types IX, XII, XIV, XVI, XIX. These collagens are associated with the fibrils formed by fibrillar collagens, and are partly responsible for the interactions between collagen fibrils and the extracellular matrix (Olsen & Ninomiya, 1999). Collagen type IX is interesting because it was the first collagen to be linked to other matrix components such as proteoglycans. It is thought to be a link between type II collagen and glycosaminoglycans (van der Rest & Mayne, 1987), but is not found in the skin (Ricard-Blum *et al.*, 2000).

Collagen type XII is found in skin and is similar to type IX. It is associated with collagen type I. Although it is not directly bound to glycosaminoglycans as is type IX, and it is not known what functionality this collagen serves (Kucharz, 1992). Type XII collagen is a homo-trimer consisting of three identical peptides and was discovered by DNA sequencing of material from chick embryo tendon (Gordon *et al.*, 1990). It shares many similar regions with type IX collagen from cartilage because it contains both helical and non-helical domains (Gordon *et al.*, 1990). These similarities suggest that like type IX collagen, type XII is associated with fibril formation, although it is yet to be found at the fibril surface (Gordon *et al.*, 1990). It has been shown that type XII collagen often appears in tissues with type I collagen, the major exception being bone (Gordon *et al.*, 1990).

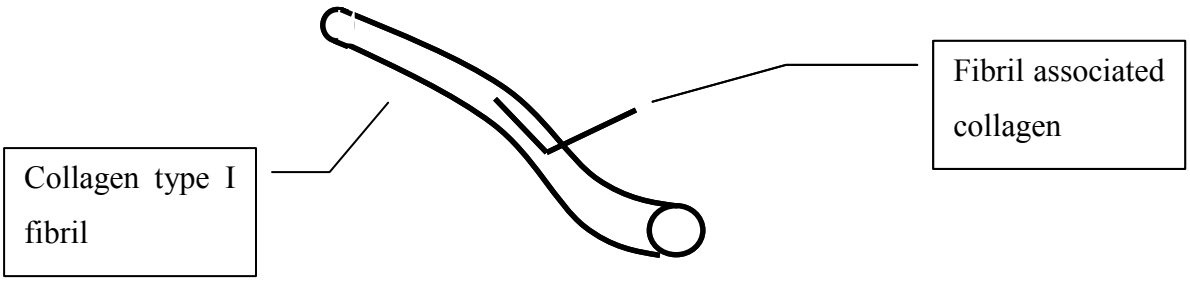


Figure 9: Fibril associated collagen. Note that a fibril associated collagen interacts with a whole fibril of collagen molecules for a portion of its single molecular length leaving a portion of the FACIT molecule exposed to the extra molecular medium.

Collagen type XII is removed during the bating stage of skin processing, because of its susceptibility to proteolysis with trypsin (Kronick *et al.*, 1991). It has been suggested that type XII collagen exists as a rigid helical collagen attached to type I fibrils with another rigid helical part extending away from the fibril as illustrated in Figure 9: Fibril (Gordon *et al.*, 1990). In bovine skin, type XII collagen is present in the papillary dermis whilst type XIV is present in the reticular dermis (Olsen & Ninomiya, 1999).

Collagen type XIV has also been shown to associate with fibrils (Bailey, 1991) and resembles type XII collagen in that it has a very similar primary sequence ("61% homology" (Kronick & Iandola, 1997)) and is associated with type I collagen fibrils (Kronick & Iandola, 1997). Collagen XIV binds to the surface of collagen fibres and to

the proteoglycan decorin. Kronick and Iandola (1997) noted a large concentration of this type of collagen in the layer of skin that becomes the grain enamel layer in the final leather. A large amount of this collagen is removed, however, during liming. Both type XII and XIV collagens are associated with the dermatan sulfate chains of decorin (Olsen & Ninomiya, 1999).

Collagen type XIX seems to only appear in a few adult tissues such as brain, eye, and testis (Olsen & Ninomiya, 1999).

2.6.1.4 Short chain collagens

This group includes types VIII and X. They are known as the short chain collagens because of the relatively short length of their triple helices (Olsen & Ninomiya, 1999).

Collagen type VIII is found in the eye and can be produced by cultured endothelial cells (Ricard-Blum *et al.*, 2000). The helical portion of this protein is interrupted twice by non-helical domains resulting in helical fragments of about one third the total molecular weight of normal collagen polymers (Kucharz, 1992). This collagen is not found in the skin.

Collagen type X is found in bones during bone development. In adults it is found in calcified cartilage and is not found outside of hypertrophic cartilage (Olsen & Ninomiya, 1999).

2.6.1.5 Basement membrane collagens

Type IV collagen is by far the most abundant collagen present in the basement membrane ((Stanley *et al.*, 1982),(Olsen & Ninomiya, 1999)). It is a major component of the basement membrane along with the glycoproteins laminin, nidogen, entactin, and heparan sulfate (Glanville, 1987). Type IV collagen does not form rigid fibrils as do the fibrous collagens. Due to the non helical domains located within the collagen molecule, type IV collagen molecules aggregate into tetramers (von Loutis *et al.*, 1984)which then form a “chicken wire” network. ((Timpl *et al.*, 1981),(Bailey & Paul, 1997)).

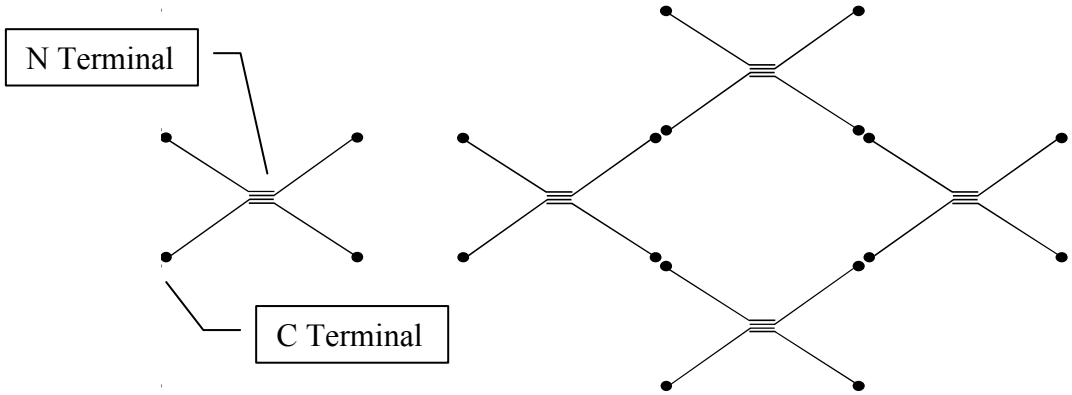


Figure 10: Type IV collagen quaternary structure from ((Timpl *et al.*, 1981); (von Loutis *et al.*, 1984),(Stanley *et al.*, 1982))

This network provides a scaffolding to support the other materials of the basement membrane in skin. The non-covalent bonds within the overlap of the N-terminal ends become stabilised by di-sulfide bonds and non-reducible cross-links (Yurchenco, 1990). The fine mesh formed by the aggregation of the tetramers has an inter-space diameter of about 40 nm. A schematic diagram of this is shown in Figure 10. Type IV collagen interacts with the cellular matrix indirectly though interaction with laminin in association with entactin (Olsen & Ninomiya, 1999).

2.6.1.6 Multiplexins

This group includes type XV and XVIII collagens. These collagens are known as multiplexins because they contain multiple short helical domains and are mostly found in basement membranes. (Olsen & Ninomiya, 1999). Their function has yet to be determined.

2.6.1.7 Collagens with trans-membrane domains (MACIT)

This group includes types XIII and XVII collagens which are cell surface molecules with multiple extracellular triple helical domains. This group is also known as the MACIT collagens in analogy to the FACIT collagens (2.6.1.3) because they are membrane associated collagens with interrupted triple helix domains. There is evidence for the presence of type XIII collagen in the epidermis and type XVII is involved in the skin disease bullous pemphigoid (Olsen & Ninomiya, 1999). In view of their location it has been suggested that these proteins are involved in cell adhesion (Olsen & Ninomiya, 1999).

2.6.1.8 Other collagens

This rather uninterestingly named group includes specialised structure forming collagen types VI and VII. Two molecules of collagen type VI form an anti parallel dimer with di-sulfide bonds, followed by the aggregation of two dimers to form a tetramer. The tetramers are the building blocks for a filamentous micro fibril structure (Ricard-Blum *et al.*, 2000) as illustrated in Figure 11. The Type VI collagen is unusual in that it has a globular structure whose function is not yet known (Chu *et al.*, 1990). The tetramer structures of type VI collagen are found close to cells, collagen fibrils, and the basement membrane indicating that they may be involved in anchoring these structures (Chu *et al.*, 1990). It has been found particularly around the hair follicle (Fukunaga *et al.*, 2006)indicating that it may play a part in the unhairing process. Type VI collagen probably plays a role in cell adhesion through protein domains that are homologous to known collagen I binding and cell binding domains (Olsen & Ninomiya, 1999). Collagen type VI, is unlike the other minor collagens found in the grain enamel described above in that it is unaffected by conventional lime sulfide processing as it remains detectable in the processed skin where it is associated with the fine fibrils connecting collagen types I, III, and V (Kronick *et al.*, 1991).

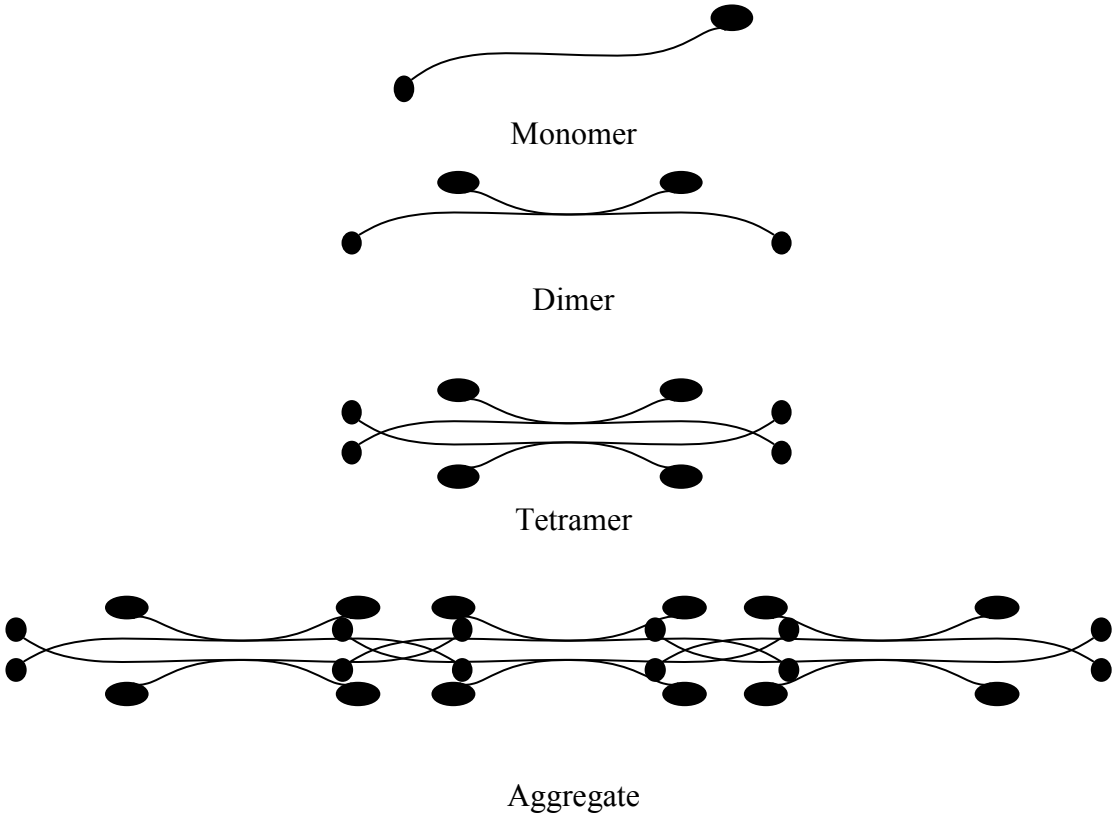


Figure 11: Hierarchy of collagen VI structure.

Although removing collagen VI is not required in conventional processing, it may be useful to remove this collagen in enzyme depilation if it is at all involved in anchoring hairs to the dermis. Alternatively, its existence in the grain enamel after processing may indicate its requirement for a quality product.

Collagen type VII is the major component of anchoring fibrils located in particular in the basement membrane (Woodley *et al.*, 2005). Although type VII collagen is characterised as being a non fibrous collagen (Bailey, 1991) it has also been characterised as a filamentous collagen (Bailey & Paul, 1997).
It is produced by both epithelial cells and to a small degree by fibroblasts (Burgeson *et al.*, 1990). Collagen type VII is one of several molecules that attach the epithelial cells to the underlying dermis. Individual molecules retain their non-helical globular region at each end in contrast to the fibrous collagens. The molecules form anti-parallel dimers through di-sulfide links along a region 60 nm in length at their amino-terminal end. The dimers then form non staggered lateral aggregates that are stabilised by inter-chain di-sulfide bonds. The quaternary structure is illustrated in Figure 12

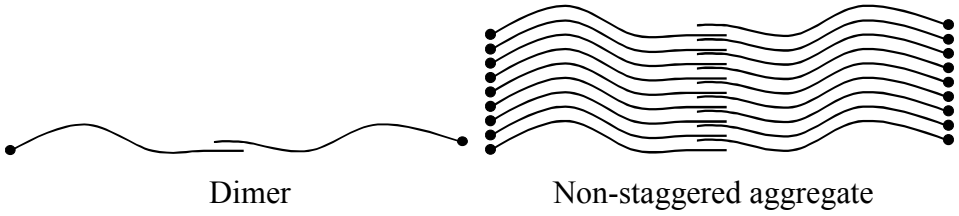


Figure 12: Type VII collagen quaternary structure

The aggregates extend through the region between the cells of the epidermis and the matrix of the dermis into the surrounding matrix, binding to plaques of collagen type IV to form a complex scaffolding (Burgeson, 1987).

The length of the type VII collagen aggregate is about 800 nm (Burgeson *et al.*, 1990) with the amino terminal groups from the different bundles overlapping at the centre. The globular regions at the N-terminal ends are excised before aggregation occurs (Burgeson *et al.*, 1990), leaving the globular end at the carboxy terminus at either end of

the quaternary structure. There is some evidence, however that the globular region at the overlapped end may become the carboxy-terminus (Olsen & Ninomiya, 1999).

The remaining regions found at each end of the aggregate are in the form of three fingers that extend into the basal membrane at one end of the type VII collagen aggregate and into anchoring plaques at the other end (Burgeson *et al.*, 1990). The globular domain interacts strongly with the type IV collagen which makes up the anchoring plaques, an interaction that is destroyed by pepsin but not so much by collagenase (Burgeson *et al.*, 1990) General proteases, which can break down the globular proteins, may therefore be valuable in enzyme depilation processes, provided that these proteins are responsible for holding in the wool. Immunological investigation has shown that collagen VII is important in the skin disease epidermolysis bullosa acquisita, a disease, which manifests itself as blisters due to the epidermis and dermis being separated at the dermal-epidermal junction. (Woodley *et al.*, 2005). as it has been shown that antibodies to collagen VII are likely to be the cause of this disease, collagen VII may be a good target for enzymatic depilation

It should be noted that type VII collagen can be cleaved by the following proteases:

- Pepsin which cleaves at a central discontinuity in the triple helix cleaving the molecule approximately in half.
- Interstitial collagenase cleaves this collagen.
- Type IV collagenase also cleaves this collagen.

2.6.2 Glycoproteins

Glycoproteins are proteins which have carbohydrate polymers covalently attached to either an asparagine, serine or threonine side chain (Hukins, 1984).

2.6.2.1 Fibronectin

Fibronectin (also known as cold insoluble globulin) is present in the dermal epidermal junction. There are many forms of fibronectin which can be grouped according to the following biochemical properties (Rollins *et al.*, 1982):

a) Fibronectins that are readily dissolved by trypsin or testicular hyaluronidase

b) Fibronectins that are resistant to enzymes and/or guanidine hydrochloride extraction

c) Those that interact with antibodies but cannot be hydrolysed by hyaluronidase
d) Those that will not react with anti-fibronectin antibodies even after various extraction or fixation techniques

Fibronectin is associated with the extracellular matrix, where it plays a role in the attachment between cells and the extracellular matrix, and can be considered to be a cell surface adhesion protein (Bongrand, 1988), (Kleinman., 1982). It may be involved specifically in cell attachment to collagen (Rollins *et al.*, 1982), which may be relevant to enzyme depilation. The form of most interest in the skin is cellular fibronectin which has a molecular weight of 220-240 kDa (Rollins *et al.*, 1982) and exists as both aggregates and as fibrils. The fibronectin molecule consists of two subunits covalently linked by a di-sulfide bridge at the carboxyl terminus. Each subunit has four globular regions one of which is involved in cell attachment while the others play a role in binding to collagen. Fibronectin binds directly to specific sites on the collagen molecule (Rollins *et al.*, 1982), and to some glycosaminoglycans at the end of the cell attachment associated globular region (Hukins, 1984) with the exceptions of dermatan sulfate and chondroitin sulfate. Because of these properties it is likely that fibronectin is involved in the attachment of cells to the extracellular matrix (Hukins, 1984).

Fibronectin carries a number of complex carbohydrate chains which give the protein some protection from proteolysis (Hynes, 1999). The molecule therefore has regions of high resistance to proteolysis and some regions that can easily be degraded by enzymes including; pronase, trypsin, and chymotrypsin. Proteolytic digestion breaks fibronectin down to separate GAG binding, and collagen binding regions (Rollins *et al.*, 1982). This susceptibility may be important in the depilation process and would explain why general wide-spectrum protease solutions can achieve depilation. If specific “fibronectinases” were available, this breakdown of fibronectin and hence depilation may be achieved without collateral damage to structural collagen. In addition it would seem that the removal of the glycosaminoglycans that attach and surround a portion of the fibronectin molecule by a broad spectrum glycosidase for example would assist in the enzymatic depilation process by enabling proteolytic enzymes access to the whole protein.

2.6.2.2 Fibulin

Fibulins are a family of proteins with similar domains. Fibulin types 1 and 2 appear to be associated with supra-molecular structure formation and/or stabilisation such as that found with elastic fibres and basement membranes (Argraves, 1999).

2.6.2.3 Laminin

Laminin is a glycoprotein that consists of three polypeptide chains. It appears as a flexible cross structure in the electron microscope with three short arms of 35 nm and one long arm of 75 nm. The long arm is thicker with a globular region at the end, while the shorter arms incorporate smaller globular regions. The three chains are held together by di-sulfide links in the region of the cross and near the globule of the long arm. Laminin contains 13% carbohydrate mainly in the form of N-linked oligosaccharides (Charonis & Tsilibary, 1990). The laminin molecule interacts with basement membrane components through both peripheral and central cross domains, and with cells at sites near the cross and end of the long arm (Yurchenco, 1990).

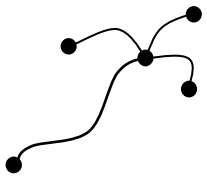


Figure 13: Schematic of laminin based on rotary shadowing images (Sasaki & Timpl, 1999).

It has also been shown to be associated with the separation of epidermis from dermis *in vivo* (Saksela *et al.*, 1981) and in association with the formation of blisters in mucous membrane pemphigoid (an autoimmune disease - characterised by the formation of sub-epidermal loss of adhesion) (Bekou *et al.*, 2005). During the formation of these blisters, laminin was observed on the edge of their dermal side (Kaminska *et al.*, 1999) indicating the possible involvement of laminin in the removal of the epidermis during skin processing. It is also possible that laminin is a major component of the grain enamel after beam house processing.

There are a number of different types of laminin all of which appear to be involved in cell adhesion interactions. Of particular interest is laminin-5 which is substantially different from other laminins having a dumbbell shape (Sasaki & Timpl, 1999). It has been shown to localise at anchoring filaments between the dermal-epidermal junction as

well as at anchoring plaques of the papillary dermis (Sasaki & Timpl, 1999). It may therefore be involved in binding collagen VII in the anchoring filaments (Sasaki & Timpl, 1999) (Woodley *et al.*, 2005).

2.6.2.4 Entactin/Nidogen

Entactin (after its discovery nidogen was found to be a degradation product of entactin) is a glycoprotein consisting of a single polypeptide chain with a molecular weight of 158 kDa. It contains about 5% carbohydrates in the forms of O-linked and N-linked oligosaccharides. Entactin has a dumbbell shape with two globular regions (diameter 6 and 4 nm) connected by a rod of 16nm in length (Charonis & Tsilibary, 1990). The dumbbell shaped molecule forms a non-covalently linked complex with laminin at about a 1:1 ratio (Timpl *et al.*, 1990). Entactin is more sensitive to proteolytic digestion than laminin, although its rod-like central portion remains relatively unaffected (Timpl *et al.*, 1990). Entactin is essential for connecting laminins to collagen IV in the basement membrane (Timpl, 1999).

2.6.2.5 Glycoproteins in hair

Formic acid has been found to remove glycoproteins from hair (Negri *et al.*, 1996). It is also likely that some of the keratinases may remove these glycoproteins resulting in a breakdown of the macrostructure of the wool fibre (Brady *et al.*, 1987).

2.6.3 Polysaccharides

Although there is no record of unhairing or dewooling caused by the sole removal of sugars from skin, and some evidence exists for amylolytic enzymes being unable to unhair in the absence of any other treatment (Yates, 1968c), there does appear to be some evidence that breakdown of sugar chains offers some additional benefit within a proteolytic unhairing procedure (Jian *et al.*, 2007), (Thanikaivelan *et al.*, 2006), (Aravindhan *et al.*, 2004). This literature and the fact that some important proteins of the extracellular matrix have attached glycosaminoglycans indicates that the sugar polymers present in the skin are of interest to the subject of proteolytic depilation.

2.6.3.1 Glycosaminoglycans

Glycosaminoglycans (GAGs) are comprised of polysaccharides, and characterised by the content of the repeating disaccharide of which those polysaccharides are comprised.

These structures are therefore more complex than glycogen but significantly less complex than proteins which typically contain about 20 different types of amino acid residues. GAGs exist as un-branched polysaccharide polymers generally consisting of a repeating disaccharide unit containing an aminoglycan and a uronic acid. The aminoglycan is either a, D-galactose, or galactosamine and the uronic acid is either D-glucuronic acid or its epimer, L-iduronic acid. In most glycosaminoglycans ester sulfate linkages may be present on some of one or both of the saccharides. Hyaluronic acid is the exception with no substituted sulfates (Rodén, 1980). Sulfate groups are found as O-sulfates or in the cases of heparan sulfate and heparin either O-sulfate or N-sulfate. On average sulfation occurs about once every disaccharide unit (Bayliss, 1984).

A number of glycosaminoglycans have been characterised, including; hyaluronic acid, chondroitin sulfate, dermatan sulfate (also known previously as chondroitin sulfate B), chondroitin sulfate C, keratan sulfate, heparan sulfate, and heparin.

The only glycosaminoglycans found in skin in significant quantities are hyaluronic acid and dermatan sulfate. The presence of other glycosaminoglycans such as heparin in skin is disputed (Mier & Cotton, 1976). Hyaluronic acid and dermatan sulfate were first detected by Mayer and Chafee in 1941 by ethanol precipitation of their barium salts (Mier & Cotton, 1976). It has been demonstrated that keratan sulfate does exist in skin by immuno-blotting using antigens against bovine keratan sulfate core protein (Funderburg & Conrad, 1989). The concentration of keratan sulfate in the skin however, is much less than that present in the cornea and makes a minor contribution to the total proteoglycans present in skin.

Glycosaminoglycans are found in greater quantities in the dermis compared to the epidermis. In each of these regions the two skin glycosaminoglycans exist in different ratios. For the most part there is about twice as much hyaluronic acid as dermatan sulfate (Kornfeld & Kornfeld, 1980). The distribution of hyaluronic acid and dermatan sulfate between species and locations on the skin around the body differs only slightly (Mier & Cotton, 1976). On a fresh weight basis the amount of hyaluronic acid in skin ranges from 119-175 ug/g whereas dermatan sulfate ranges from 62-94 ug/g.

2.6.3.1.1 Hyaluronic acid

Hyaluronic acid contains repeating disaccharides of D-glucuronic acid and 2-acetamido-2-deoxy-D-glucose (also called N-acetylglucosamine).

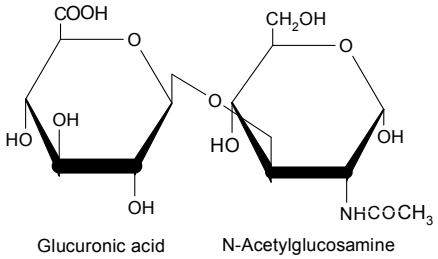


Figure 14: Hyaluronic acid disaccharide unit

A distinguishing feature of hyaluronic acid is the lack of sulfation, which means that it is much less acidic than dermatan sulfate, providing a means to distinguish between the two (Mier & Cotton, 1976). Hyaluronic acid has a very high molecular weight (in the millions) (Toole, 1999) and forms a large tangled weave that traps a large quantity of bound water. This results in a material through which it is difficult for large molecules to pass (Toole, 1999). The removal or breakdown of hyaluronic acid is therefore likely to help enzymes such as proteases to reach their substrates.

2.6.3.1.2 Dermatan sulfate

Dermatan sulfate is made of repeats of L-iduronic acid and 2-acetamido-2deoxy-4-D-galactose (also called N-acetyl-galactosamine) or galactose, either of which may or may not be O-sulfonated. The sulfation results in a highly charged highly acidic proteoglycan (Alexander *et al.*, 1986). About 10% of glucuronic acid is not converted to iduronic acid, in particular in the region of the glycosamine glycan closest to the protein backbone (Bettelheim, 1971).

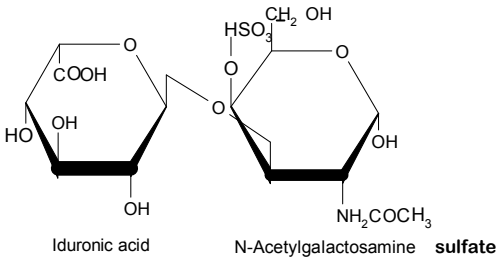


Figure 15: Dermatan sulfate disaccharide unit

These polysaccharide chains are linked to the peptide backbone of proteoglycans at a number of points. As the polysaccharide is normally much longer than the peptide chain, there are long loops of polysaccharide between each peptide linkage. Three types of linkages to peptides are known in connective tissue:1) O-glycosidic linkage between D-xylose and the hydroxyl group of serine, 2) N-glycosidic linkage between N-acetyl-glucosamine and the amide group of arginine, and 3) O-glycosidic linkage between N-acetyl-galactosamine and the hydroxyl group of serine or threonine (Rodén, 1980).

2.6.3.2 Glycogen

Glycogen makes up only a very small part of the polysaccharides found in skin (less that 0.1% total dry weight (Mier & Cotton, 1976)). It is found in the sweat glands and hair follicle where it can reach 2.8% on a dry weight basis (Mier & Cotton, 1976). It appears to be involved in the sweating and hair growth processes. The glycogen found in skin is essentially identical to the glycogen of the liver. It appears that since the glycogen in skin represents only 10 minutes of energy supply required, it is unlikely to be present as an energy store. It is more likely that glycogen is present as a source of specific glycogen derived molecules required during active synthesis. It is therefore unlikely that glycogen has any impact on the process of enzymatic depilation after slaughter.

2.6.4 Proteoglycans

Proteoglycans (formerly known also as mucopolysaccharides) are a class of proteins with one particular post-translational modification. All proteoglycans have chain(s) of glycosaminoglycans (GAGs) attached. The core protein can be anywhere from 10 kDa to 400,000 kDa and the number of GAG chains can be as few as 1 or more than 100 (Lander, 1999). Proteoglycans are characterised by their attached GAGs (Lander, 1999).

Proteoglycans make up a portion of what is known as the ground substance. Ground substance is the extracellular material found in skin especially between the dermis and the epidermis. For the most part proteoglycans consist of polysaccharide chains of glycosaminoglycans attached to a protein backbone (Bayliss, 1984). For example 80-100 chains of chondroitin sulfate and 30-40 chains of keratan sulfate attached to a core protein are found in the proteoglycans of bovine nasal septum cartilage (Bayliss, 1984).

Their un-branched structure and limited type of monosaccharide fits well with their roles as binding molecules (Mier & Cotton, 1976). In particular they are found in roles where the specific shape of the molecule is not important (Mier & Cotton, 1976). A few proteoglycans do have highly specific functions. For example heparin has anti-coagulating properties and other proteoglycans display cell binding properties(Lander, 1999).

2.6.4.1 The role of proteoglycans in skin

The attached glycosaminoglycans contain a large number of sulfate and carboxylate groups which become dissociated at physiological pH. The ionic forms of the polysaccharides then exist as a negatively charged molecule in solution surrounded by a cloud of counter ions (Myers *et al.*, 1984). The network of glycosaminoglycans formed by chains of polysaccharides attached to protein backbones interact with each other and form strong networks within the collagen matrix (Myers *et al.*, 1984). It is also likely that some are covalently bound to the collagen (Mier & Cotton, 1976). The affinity of water for glycosaminoglycans is so strong that the volume of a hydrated molecule may be 1000 times that of its non-hydrated form (Myers *et al.*, 1984). The swelling force of this hydration approaches 0.35 Mpa (greater than 3 times atmospheric pressure) and is a function of the fixed charge density. *i.e.* the concentration of the bound sulfate and carboxylate groups present in the proteoglycan. The proteoglycans therefore provide a support for the scaffolding of the collagen network analogous to balls of glass in a piece of macramé. In addition to their structural role glycosaminoglycans act as a medium through which metabolites pass to reach cells in the skin (Mier & Cotton, 1976). Proteoglycans also play a major role in binding specific metabolites, sequestering them from other ligands or holding them for specific ligands to interact with (Lander, 1999). The functions of proteoglycans are summarised below:

1. Regulation of the activity of proteases and protease inhibitors
2. Regulation of cellular responses to growth factors
3. Regulation of cell-cell and cell matrix interactions
4. Regulation of extracellular matrix assembly and structure
5. Immobilisation of diffusible molecules

(Lander, 1999)

Of the most interest are functions 3 and 4. Proteoglycans with function number 3 possibly being important in the depilation process. The removal of particular proteoglycans may therefore be required before enzyme depilation can commence. Immobilisation of diffusible molecules (function number 4) may also be important as certain proteoglycans (e.g. decorin) may affect the processing of skin after depilation (Kronick & Iandola, 1998).

The exact role of skin proteoglycans depends not only on the nature of the glycosaminoglycan side chain but also on the core protein to which it is attached (Reichardt, 1999). The core proteins of proteoglycans contain different functional regions including highly specific polysaccharide binding regions (lectin like domains), or immunoglobulin-like domains (Reichardt, 1999). Such domains are involved in cell ligand binding (Reichardt, 1999).

Both glycoproteins and proteoglycans can have a number of domains with distinct functions. The same or functionally similar domains can exist on a number of different proteins present in the extracellular matrix. A list of domain types and examples of the proteins that possess those domains have been described (Engel & Bozic, 1999).

2.6.4.2 Specific proteoglycans

The different proteoglycans can also be grouped according to their core protein similarity as:

1) Small interstitial proteoglycans
2) Large chondroitin sulfate proteoglycans such as aggrecan
3) Cell surface proteoglycans

Some other proteoglycans such as perlican, agrin, or phosphocan cannot be grouped into these families (Lander, 1999).

2.6.4.2.1 Biglycan

Biglycan is a member of the leucine-rich repeat (LRR) protein family and is composed of a 38kDa core protein that is substituted with two glycosaminoglycan chains consisting of either chondroitin sulfate or dermatan sulfate, with DS being more abundant in most connective tissues on N-terminal Ser-Gly sites. The core protein contains ten leucine rich repeats flanked by disulfide bond stabilized loops on both sides

(Fisher *et al.*, 1991),(Neame *et al.*, 1989). It is found in the epidermis but not the dermis. It binds to type I and V collagen and can be found at the edge of the lamellipodia of migrating endothelial cells (Fisher, 1999a).

2.6.4.2.2 Decorin

Decorin is a small proteoglycan that is associated with collagen fibrils. The core protein is about 38 kDa and it has one dermatan sulfate polysaccharide chain (Fisher, 1999b). The dermatan sulfate side chain is about 16 kDa (Kronick & Iandola, 1998). Immuno-histochemistry has been used to determine that the protein core of decorin remains associated with the collagen fibril throughout conventional lime sulfide processing (Kronick & Iandola, 1998). Decorin is probably associated with collagen fibril formation (Fisher, 1999b).

2.6.4.2.3 Versican

Versicans are a family of proteoglycans which have a number of chondroitin sulfate chains attached. They do not appear to be directly involved in the actual act of adhesion and so are unlikely to be involved in the process of enzyme depilation. However, they may be involved in the modulation of cell adhesion (Zimmermann, 1999).

2.6.5 Other Proteins

2.6.5.1 Elastin

Elastin is the main extensible protein which allows reversible stretching of the skin (Mier & Cotton, 1976). The elastic recoil of elastin is provided by the hydrophobic domains within the protein. When elastin is stretched the hydrophobic regions come into contact with water decreasing the entropy. Then, when the tension is released, the hydrophobic regions re-aggregate and expel the water, and the entropy increases which drives the movement. (Mecham, 1999). Elastin is an insoluble protein that always appears in conjunction with collagen. It contains the cross linking amino acids desmosine and isodesmosine which are specific to only elastin, and allow a convenient means of measuring its concentration. The amino acid composition of human elastin can be found in Mier and Cotton 1976; p 86; Table 4.3. The amino acids involved in cross linking are illustrated in Figure 16.

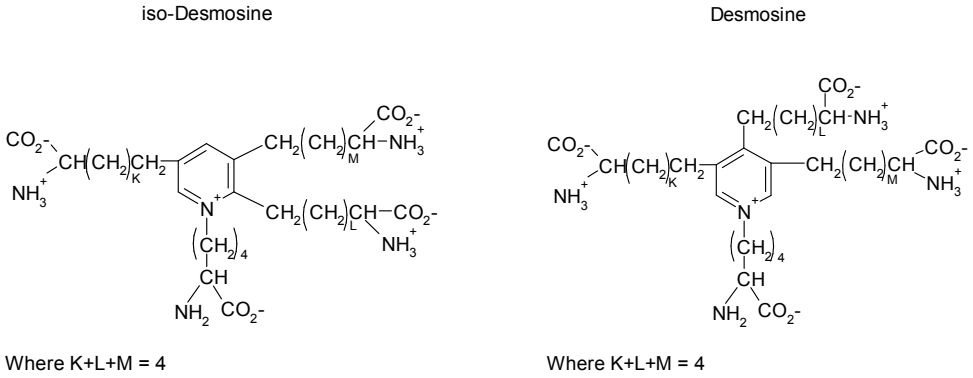


Figure 16: Cross linking amino acids of elastin

Desmosine and isodesmosine as illustrated in Figure 16 are formed from linking lysine amino acid residues (Montagna & Parakkal, 1974). Elastin can be histologically viewed using the stains orcein, or resorcin and fuchsine. It appears as a web-like, branched fibrillar structure, the fibres having the form of an amorphous material surrounded by crystalline microtubules (Mecham, 1999). Very fine fibres branch from the main web of fibres in the dermis and extend into the epidermis as barely resolvable "fibrelets" (Montagna & Parakkal, 1974). It is possible that elastin fibres are also involved in anchoring the epidermis to the dermis. If enzymes are to achieve depilation (un-assisted by sulfide) then some destruction of the elastin network may be inevitably required. Alternatively given their location throughout the grain it is possible that damage or removal of elastin may adversely effect the resulting product after depilation. More elastin appears within the grain than deeper in the skin, with some elastin reappearing at the bottom of the corium. Elastin comprises about 2.7% of the dry matter in the grain layer (Keller & Heidemann, 1989). Previous work on the importance of elastin has mostly focussed on bovine hides (Webster *et al.*, 1987). Many characteristics have been attributed to elastin removal from leather including:

- Increased flatness - (Stanley, 1994)
- Reduced flatness - (Allsop *et al.*, 2005); (Edmonds, 2002)
- Increased softness - (Ornes *et al.*, 1960)
- Increased area - (Alexander *et al.*, 1991)
- Increased veininess- (Alexander *et al.*, 1991)
- Drawn grain - (Ornes *et al.*, 1960)
- Reduced area - (Allsop *et al.*, 2005); (Edmonds, 2002)

It is quite clear from these reports that our "knowledge" of the true effect of elastin removal is contradictory to say the least. Also much of the work done overseas is limited only to bovine hides. It is known that bovine material has different quantities of elastin in comparison to ovine skin (Cooper, 1998a); (Lowe *et al.*, 2000) and that the distribution of that elastin within the hide is also different compared to sheep skin. It is not reasonable therefore to assume that the effects noted for removal of elastin in bovine hide will apply to sheep skins. This may explain some of the contradictions noted above but does not tell us what to expect for the removal of elastin in New Zealand sheep skin stock. Since elastin appears important in processing and since it appears in the grain surface of raw skin it would be prudent to investigate its importance in enzymatic depilation.

2.6.5.2 Keratin

Keratins are characterised by high cysteine content. The strength of wool, insolubility, and resistance to acid attack can be attributed to the di-sulfide cross links of cysteine residues. In prekeratin, only a small number of di-sulfide links have formed, making it susceptible to digestion by trypsin (Matoltsy, 1965).

Keratins are also characterised by being polydisperse. Many different genes for keratin are known. Some produce a "soft" keratin such as that found in the outer root sheath and others produce a "hard" keratin such as that found in the hair cortex (Limat *et al.*, 1991).

A coiled-coil structure for keratin molecules has been proposed (Mier & Cotton, 1976) where single polypeptide chains form into right handed helices. These in turn are coiled together into a left handed helix. The coiled coils are then aggregated into micro-fibrils which in turn are cemented into a sulfur rich protein matrix. This matrix is globular in nature with intermolecular di-sulfide bridges (Mier & Cotton, 1976).

2.6.5.3 Tenascin

The tenascins are a family of proteins with similar domains. Tenascins bind to heparan sulfate proteoglycans. Tenascin-x and tenascin-c are both found in skin. Tenascin-c is anti-adhesive in that its presence results not in cell adhesion but cell rounding (Chiquet-Ehrissmann, 1999). It is unlikely that this protein is involved in the depilation process.

2.6.5.4 Vitronectin

Vitronectin is another cell adhesion type protein which binds collagens and heparin (Hall, 1999).

2.6.5.5 Trichohyalin

Trichohyalin forms granules in the hair cells that are visible under the microscope. The proteinaceous granules are associated with the differentiation of hair cells. The number of trichohyalin particles increases until hardening begins at which point they become undetectable. It is likely that these particles become part of the hardened protein in the permanent zone of the hair (Orwin, 1979). It is unlikely that particles within the hair would be associated with a depilation mechanism that occurs outside the layer of the cortex as described in detail in a later section (2.7).

2.6.6 Lipids

There are three main types of lipids in the skin follicle and hair;

i) The bound lipids that occur in cell membranes.

ii) Lipids associated with sebum, which are formed in the sebaceous glands and coat the fibre prior to its exit from the follicle mouth. These lipids are known as lanolin or wool grease.

iii) Layers of lipids that are covalently bound to the surface of the cuticle cells. These lipids can be removed using methanolic KOH unlike the lipids associated with the cortical cell membranes.

Lipids also make up a large portion of the dry matter in the skin, the majority of which exist at the grain corium junction and may act as a barrier to enzyme penetration. The focus of this work is on the proteolytic process of depilation so the details of lipids in skin were not examined in more detail. It is possible that the presence of fat could impact the enzyme depilation process by acting as a barrier to depilation and this is partly investigated further in later sections (4.3.2.3).

2.6.7 Skin components - Summary

- Collagens are the most important component of the skin matrix in terms of leather making. While collagen type I is by far the most abundant protein in skin it is relatively stable. The minor collagens which bind to collagen I may, however, have

a significant impact on the nature of the final product, and examination of their fate during enzymatic depilation would therefore be prudent

- Elastin – appears to be involved in the "looseness" fault and should therefore be conserved during any processing.

Although outside the scope of this project non-protein components of possible future interest have been identified as follows:

- Lipids may act as a barrier to enzyme penetration
- Proteoglycans – while somewhat water soluble, surround the proteins which are targets for enzyme depilation and may therefore act as a permeable barrier to depilation.

2.7 The enzyme depilation mechanism

Enzyme depilation is technologically challenging compared to sulfide based fellmongery. The positive prospects of enzyme depilation ameliorate or eliminate many aspects of conventional sulfide based processing as explained in section 2.3.
The problem with current enzyme depilation processes include: risk of damage from bacterial growth during the enzyme holding period, including risk of damage from autolytic enzymes; strict requirements for temperature control; excess removal of elastin which has been shown to result in loose leather; skin irritation for the handlers of the processed skins; grain surface damage through the over action of the depilation enzyme preparation (Passman, 1997).

Enzyme depilation is so different from the conventional processing regime that a successful enzyme depilation product would revolutionise fellmongering. Enzymatically depilated skins would be fundamentally different from the skins depilated by sulfide so it is possible that preservation and tanning methods might also be required to be modified.

2.7.1 Structural aspects of enzyme depilation

2.7.1.1 Diffusion through skin

The skin acts as a barrier to external materials so that the penetration of chemicals into the skin from the grain side is relatively slow compared to penetration from the flesh side. However, the distance to the wool root from the grain side is less than from the flesh side. The reason for the difference in diffusion rate between the two sides is because of the protective layer of keratinaceous material at the grain side surface (Cantera *et al.*, 2005). It was found that by shaving the wool off and then applying depilatory enzymes the rate of depilation by enzymes was greatly increased. (El Baba *et al.*, 2000). This was attributed to the access created down the core of the hair shaft to the site of activity. It was also proposed, that provided the enzyme solution does not damage the wool, grain side application might dramatically reduce depilation time (Yates, 1969a).

Thicker skins took longer to depilate and approximately 70% of the depilation time was related to the time taken for the enzymes to diffuse to the site of activity (Yates, 1969a). It was also noted, however, that depilation time did not correlate with skin thickness. It was also observed that skin thickness does not relate to porosity to air (Allsop & Passman, 2003). However porosity was found to correlate with the amount of non-collagenous materials removed during processing to the pickled stage. These observations suggested that while skin thickness is important, the composition of the skin and its underlying structure may have a greater effect in determining the rate at which enzymes can diffuse. This concept is partly investigated in a study of variability of dewooling carried out in section (4.2).

It was also found that the more active the enzyme preparation, the less important the rate of diffusion (Yates, 1969a). Specifically, the decrease in depilation time for enzymes when injected directly to the wool root was less marked when highly active enzymes were used compared to those with lower activity.

A number of treatments have been tested with the aim of increasing the rate of diffusion to decrease the enzyme depilation time (Yates, 1969a). Treatments included:

NaCl extraction of the skin; cetyl trimethyl ammonium bromide (CTAB); dimethyl sulfoxide (DMSO) wash; and ultrasonic treatment. None of these were found to significantly decrease the depilation time although it is possible that some may have had a positive effect on enzyme depilation. Sodium sulfite, surfactants, urea, and terpenes have also been used to enhance penetration of the enzyme through the skin as they had previously shown promise in aiding drug delivery through the epidermis (Cantera *et al.*, 2005),(Edmonds, 2007).

2.7.1.2 Stages of depilation

Enzyme depilation appears to occur in three stages (Brady *et al.*, 1989a):
i) Movement of enzyme to wool root; ii) Initial fast reduction in depilation load; iii) Slower reduction in depilation load down to the "ready-to-pull" load.
The stages of enzyme depilation on pig skins have been examined using a number of depilatory enzymes and a histological staining technique (Jin *et al.*, 1996). It was found that depilation began with the separation and destruction of the outer root sheath (ORS) followed by separation of the papilla from the hair bulb. The ORS was the structure that was most easily destroyed during enzyme depilation, while the inner root sheath remained intact throughout the process. It was therefore concluded that destruction of the ORS was a primary requirement for enzyme depilation (Jin *et al.*, 1996). However, enzymes that solely separated the ORS from the surrounding substructure did not achieve depilation in isolation. A four phase process has also been suggested (Cantera *et al.*, 2005). The four phases involve a modification of the epidermis to enable passage of the enzyme depilatory reagent, diffusion of the enzyme to the hair root, action of the enzyme on the hair root to release the hair from the shaft, and finally a "gentle" action on the collagen structure to open it up.

2.7.1.3 Impact of enzyme depilation on macromolecular structures

2.7.1.3.1 Basal layer

It was claimed that the site of enzyme depilation is immediately beside the basement membrane at the dermal-epidermal junction (Stirtz, 1965); (Cantera, 2001d). A study with transmission electron microscopy has shown that a light region in the middle of the basal membrane is destroyed during enzyme depilation of pigskins. The destruction of this region, it is claimed, is the cause of enzyme depilation (Wang *et al.*, 2005). It has also been claimed that the adhesion proteins between cells in the outer root sheath and

basement membrane are the target of this activity (Yates, 1972). Another possible model for the enzymatic depilation process has been proposed in which the basement membrane of the dermal-epidermal junction is destroyed through the action of general proteases on adhesion proteins (Brady *et al.*, 1989b). It was proposed that some strands of the three-dimensional web like structure of adhesion and support proteins are initially broken by proteolytic enzymes. As alternate enzymes with additional unique specificities are added to the mix more strands in the web are broken until a point is reached when depilation can occur.

2.7.1.3.2 Cell dispersion

It was found that the activity of cell dispersion by enzymes correlated well with depilatory activity as measured by the dispersion of mouse embryo cells examined under the microscope (Yates, 1969a). This method was not quantitative however and so could only be used as a rough ranking tool. Trypsin has been commonly used in the past to disperse cells (Yates, 1969a). It has also been stated, however, that trypsin does not completely disperse the cells, leaving cells with thin fibrous attachments remaining (Brady *et al.*, 1989a). In fact trypsin is known to be a poor depilation agent most probably because of its high specificity(Yates, 1968d).

2.7.1.3.3 Root sheaths

An examination of the root sheaths of wool (described in detail in section 2.5.4.2) during enzyme depilation using a SACPIC stain (Yates, 1968e) showed that the effects of the enzyme on skin structure occur in the following order:
Firstly the ORS and epidermis separate from surrounding structures and some lower ORS cell are destroyed. However, it was suggested that destruction of the IRS is not required for depilation to occur (Yates, 1968e). Next cells surrounding the prekeratinised zone are completely destroyed resulting in the tapered appearance of wool fibres that can be pulled after the process has proceeded sufficiently (Yates, 1968e).

2.7.2 Chemical mechanism of enzyme depilation

It would be useful to follow the enzyme depilation processes microscopically using immuno-histo-chemical techniques for the different components of the basement

membrane in order to dissect the process (Brady *et al.*, 1989b). This therefore was the subject of investigations carried out in section 5.4.

Previously eighteen different enzymes were tested against a diverse range or substrates and their ability to depilate (Yates, 1968b). The only substrate tested that showed a correlation between hydrolysis and depilation was freeze-dried blood plasma. This may have been related to the presence of fibronectin in the plasma. Activities against other substrates were trialled and found to have no correlation with enzyme depilation. These substrates included casein, hyaluronic acid, and a number of extracts of collagen. Other authors have also investigated the relative activities of potential leather processing enzymes (Galarza *et al.*, 2004).

Hyaluronidase activity was measured viscometrically and an inverse relationship with depilation was found (Yates, 1972). These tests at pH 6.5 a different pH from that usually used in depilation (usually pH > 9.0, refer to section 2.7.2.3). Presumably a physiological pH was used to ensure that all enzymes remained active during testing.

The depilatory activities of the crystalline forms of a number of different enzymes were correlated with their activities against various substrates (Yates, 1972). The enzymes used spanned the following activities: hyaluronidase, chondroitinase (measured as sulfatase activity), β-glucuronidase, collagenase, elastase, and esterase activity.
No correlation was found between any of these activities and depilatory activity. It was concluded therefore that specificity is not important in depilation and the only requirement for successful depilation is a broad-spectrum endopeptidase (Yates, 1972).

2.7.2.1 Specific proteins and the enzyme depilation mechanism

2.7.2.1.1 Collagen types I and III

It was previously noted that maintenance of collagen structure is of utmost importance in leather making. Damage to the collagen at the grain surface is detrimental to the final leather as it results in a dull finish (Wang *et al.*, 2005). The grain surface is equivalent to the top most layer of the dermis and consists of large quantities of the thin fibril collagen type III (Brady *et al.*, 1989a). Since collagen type III is more susceptible to general proteolytic attack than type I, it is likely that damage to the grain surface

observed during enzyme depilation is caused by destruction of the type III collagen by general proteases and not collagen type I which is relatively resistant to the proteolytic attack (Brady *et al.*, 1989a). It was stated earlier that collagen III is found around the outside of fibrils and is particularly prevalent in small fibrils (due to the larger surface area). If destruction of collagen III were found to be the cause of dull grain during enzymatic depilation this would therefore likely manifest itself as a change in the size or appearance of the surface fibrils themselves.

2.7.2.1.2 Collagen type IV

Due to its location at the basal layer, type IV collagen could be considered a likely target for specific attack. The enzyme "Dispase", which supposedly neither attacks type I nor III but targets collagen type IV below the epidermis, was trialled for depilation (Paul *et al.*, 2001). When used, the enzyme resulted in a sloughing off of the epidermis in a sheet. It did not damage the grain surface although some fine hairs were left behind. However, it was found that Dispase caused damage to the elastin network, which may be detrimental to the final leather product depending on its use. Clearly the sloughing of epidermis is not the only requirement for complete depilation.

2.7.2.1.3 Keratin

Work on the breakdown of feathers by keratinolytic micro-organisms and enzymes have been extensively reviewed (Onifade *et al.*, 1998). Recently enzymes have been isolated from dermatophytic micro-organisms which live on or within keratin substrates in a parasitical relationship; unfortunately the mechanism of keratin catabolism in these organisms remains elusive. It has been found that keratin can be degraded by enzymes from certain strains of *Streptomyces* (Brady *et al.*, 1987) (Nakanishi & Yamamoto, 1974) (Gehring *et al.*, 2002). When these "keratinases" have been used for depilation the process has been found to be incomplete, with fine hairs remaining on the skin. It seems unlikely that a true keratinase would leave the fine hairs intact while allowing larger hairs to be removed, so it is more likely that these mechanisms of hair removal are not in fact related to the destruction of the keratin protein itself but to some other process Other authors have found similar keratinase activities in other microbial strains (Cheng *et al.*, 1995). It was found that feather meal could be hydrolysed by general proteases but not completely. In addition the enzyme was not keratin specific but also hydrolysed collagen and elastin. Furthermore, another keratinolytic enzyme was found

to be very active against feather keratin (and other non keratin proteins such as casein and gelatine) but did not hydrolyse wool or human keratin (Böckle *et al.*, 1995). Interestingly, the micro-organism itself was significantly more successful in breaking down feather substrates than the isolated enzyme which only achieved 10% dissolution of the substrate. This suggests that the microbe may produce a suite of enzymes that act in concert to breakdown keratinaceous materials.

In most instances wool is degraded by "keratinases" into the keratinaceous cells of the cortex and cuticle (Brady *et al.*, 1987). These cells are most likely released due to destruction of the non-keratinous cementing materials found between cells and not because of a true keratin degrading enzyme or one would expect the cells to also breakdown. It is therefore likely that many of the enzymes that are claimed to have keratin degrading ability are able to degrade wool fibres due to their general proteolytic activity or specific activity against the cementing substances of the wool fibre, or a combination of both.

In addition it has been found that the destruction of keratin is not a requirement for enzyme depilation (Foroughi *et al.*, 2006); (Yates, 1968b). It has been found that destruction of hard keratin is not only difficult to achieve but is unrelated to depilation, and that the breakdown of soft prekeratin which contains much fewer di-sulfide bonds is more likely to be beneficial (Brady *et al.*, 1989a).
In conclusion keratinases are not of value in depilation as they are non-specific, not able to completely degrade keratin (or specifically wool keratin), and not required to achieve depilation.

2.7.2.1.4 General proteases

Proteases with the best depilation attributes have tended to be relatively non-specific as far as their substrate specificity is concerned (Yates, 1968d). The implication is that certain protein cleavages are required for depilation and that some of these can only be achieved by broad-spectrum proteases. Purified enzyme preparations with high specificity (*e.g.* purified trypsin) have in the past proved to be poor depilation agents (Yates, 1968c). This is likely because a number of specific hydrolytic activities are required either in concert or in a defined order. It has been claimed that a successful depilation agent is the one that breaks the greatest number of possible peptide bonds

(Yates, 1972). It is also claimed that the action of a broad range of proteases active against a number of different proteins involved in attachment can cause the required loosening (Brady *et al.*, 1989b). Therefore by including multiple proteases in an enzyme formulation, depilation should be easily achieved. Unfortunately when multiple general proteases are used the collagens and elastin are also at much greater risk of being attacked.

However, since many proteins share a number of protease recognition sites (Engel & Bozic, 1999) it may be impossible to develop a protease cocktail to attack specific proteins without attacking others that should remain intact. This presents a serious problem and suggests that proteases which are more targeted than currently used may still cause unwanted damage. Finding a protease that effects depilation whilst causing no damage may be difficult and may require a cocktail of proteases which are highly specific to individual sequences found on only those proteins of interest.

2.7.2.2 Carbohydrates

Previous studies investigating the impact of glycosidase activity on depilation were carried out with crude enzyme formulations which may have contained proteolytic activity and it is quite possible that the depilation activity observed was related to the proteolytic activity of these contaminants and not to the glycosidic activity itself (Brady *et al.*, 1989a). It was noted earlier that the presence of proteoglycans in skin may act as a barrier to enzyme depilation. It is therefore possible that the additional help of a glycosidic enzyme may assist a proteolytic enzyme depilation regimen. A combination of protease and amylase have also been used where it was claimed that the "opening up" of fibres as achieved by the amylase while fibre release was carried out by the proteolytic enzyme (Thanikaivelan *et al.*, 2007).

Enzyme depilation was examined using periodic acid Schiff (PAS) staining (Yates, 1968e). The intensity of this stain is directly proportional to the 1:2 glycol groups of monosaccharides present (Walsh & Jass, 2000). A PAS stained layer that remains unaffected throughout enzyme depilation is situated adjacent to the region of depilation and enzymes are required to move through it to the site of activity (Yates, 1968e). This region was identified as the basement membrane and it was observed that separation of the cells in the epidermis and outer root sheath from this basement membrane seemed to

be important in depilation (Yates, 1968e). The implication here is that removal of glycosaminoglycans is removing a barrier to enzyme depilation

However, it was noted that the PAS stained membrane described above lost its affinity for the stain during enzyme depilation and that this region was not affected by hyaluronidase or alkaline solutions (Brady *et al.*, 1989a). This indicates that the membrane may have high concentrations of dermatan sulfate proteoglycans which are changed during the enzyme depilation process. It is therefore probable that removal of dermatan sulfate in addition to hyaluronic acid may aid the enzyme depilation process.

2.7.2.2.1 Glycosaminoglycans – Hyaluronic acid

It has been suggested that the removal of proteoglycans is of importance to enzyme depilation (Cantera, 2001d). Amylolytic depilation has shown that removal and elimination of proteoglycans may be important in the enzyme depilation process. However hyaluronidase activity as measured by reduction in viscosity of a hyaluronic acid substrate was not found to correlate directly with depilation activity (Yates, 1968d). During conventional processing hyaluronic acid is removed during the soaking stage, while the wool is still tight (Alexander *et al.*, 1986) indicating that this substance is not responsible for holding the wool root in place. Its removal may, however, assist the penetration of other enzymes to their targets. This is illustrated by reduced “depilation load” when skins are de-swelled after washing (O'Flaherty *et al.*, 1956).

2.7.2.2.2 Glycosaminoglycans – Dermatan sulfate

Dermatan sulfate is removed during the alkaline stages of conventional processing (Alexander *et al.*, 1986), though not removed by simple soaking. It is associated with proteoglycans and as it may influence the binding of epidermal cells to the underlying structures, is an obvious target for enzymatic depilation mechanism. No correlation between sulfatase activity on dermatan sulfate and the quality of depilation was found (Yates, 1972). Nevertheless the breakdown of dermatan sulfate and subsequent destruction of the protein backbone of adhesion glycoproteins outside the papilla would have been more useful.

2.7.2.2.3 Glycoproteins

It was found that the destruction of specific glycoproteins was important, as their removal helps release the hair bulb from the papilla (Jin *et al.*, 1996). However, these researchers go on to state, that breakdown of other glycoproteins outside the papilla is incidental to depilation.

2.7.2.3 Influence of pH (alkali in particular)

It has been stated that skin is impermeable to enzymes at neutral pH and requires the addition of alkali to open the structure sufficiently for penetration to occur (Cantera, 2001a). It can therefore be inferred that previous work carried out at acid or neutral pH is not complete and may benefit from repetition at higher pHs. Alkali plumping during processing is reported to be a requirement for obtaining good leather. (O'Flaherty *et al.*, 1956). It has also been found that some enzyme preparations achieve a better quality product when used in a depilatory applied directly to raw skin (Felicjaniak, 1985) and some only achieve depilation after an alkaline pre-treatment of the skin (Simoncini, 1987). These observations have led to the popularity of seeking depilation enzymes amongst alkali stable proteases (Dayanandan *et al.*, 2003); (Passman, 1997). It has been found that although a pre-treatment in alkaline conditions can improve depilation, it is not a requirement (Brady *et al.*, 1989b). Alkaline treatment may therefore alter the molecules in the skin in such a way as to predispose them to attack by broad-spectrum proteases. However, if different proteases, are used this predisposition may not be required. It may be that the use of an alkaline stage prior to or during the depilation process removes alkaline soluble material not otherwise removed by the depilatory enzyme. The removal of this material may directly impact the loosening of the wool fibres or alternatively it may remove material that acts as a barrier to the depilatory enzymes.

Alkaline treatment during processing also impacts on the final leather product due to the swelling imparted to the collagen at higher pHs (O'Flaherty *et al.*, 1956). Under conventional processing, an alkaline environment is used to swell and plump the collagen so the fibres are opened up. Without some opening up action the fibre structure remains compact producing a hard leather (Brady *et al.*, 1989b), but processes in which the pH is not raised as high as it is in conventional processing are claimed to result in

flatter pelts with enhanced sensory properties due to the reduced level of swelling occurring at lower pH (Edmonds, 2001), (Thanikaivelan *et al.*, 2000a)

2.7.2.4 Salts

It has been found that pre-treatment with sodium chloride effects the epidermis and skin in such a way as to facilitate enzyme depilation (O'Flaherty *et al.*, 1956). In particular lyotropic agents were reported to reduce the "depilation load" in a reversible manner. Lyotropic agents such as salt have the effect of de-swelling proteoglycans by withdrawing water through the osmotic effect (Cantera, 2001d). The de-swelling may temporarily open up the structure to release the hair. This opening up may also improve the access of the depilation agents to the follicle which would explain why salts appear to help some depilation enzymes. Salt solutions also help to remove soluble proteins thereby directly removing supporting material from the follicle allowing enzymes access to it.

2.7.3 Enzyme assisted depilation

A number of authors claim to have achieved depilation with an enzyme product (Bienkiewicz, 1983), (El Baba *et al.*, 1999), (Money, 1996), (Fadal & Speranza, 1996) (Christner, 1988), (Alexander, 1988), (Boast *et al.*, 1989), (Liang-jun, 1987), (Smidek & Heidemann, 1987), (Alexander, 1987), (Thanikaivelan *et al.*, 2000a), (Thanikaivelan *et al.*, 2000b). However, care should be taken when reviewing these reports, as close inspection of the procedures used shows that, despite the titles of some articles, the processes and products described do not achieve successful depilation in the complete absence of sulfide salts. Either the depilation is incomplete and requires a sulfide based "reliming", or damage to the grain surface occurs. Most claim a large reduction in the amount of sulfide used in comparison to conventional sulfide based processing but few of the reported 100% sulfide free procedures resulted in a cleanly depilated material with no damage to the grain. Nevertheless it is interesting to note that it has been reported that quantities of sulfide, normally insufficient to achieve depilation alone, can depilate in the presence of enzymes. It was noted earlier that the sulfide mechanism of depilation involves destruction of the wool shaft whereas enzyme depilation is likely involve breakdown of the molecular structure of adhesion proteins and the cellular root sheaths by the enzyme. Therefore, instead of viewing the process as an enzyme

enhanced sulfide depilation as in previous work, it may be interesting to examine this mechanism in terms of an sulfide enhanced enzyme depilation.

It has been previously reported that sulfydryl binding inhibits cell adhesion (Grinnell, 1982). It therefore follows that di-sulfide bonds, may be required to maintain the integrity of cellular adhesion. If sulfide addition enhances enzyme depilation by causing breakdown of cell adhesion then selectively degrading other parts of those same adhesion proteins via methods other than sulfide addition, may also assist enzyme depilation.

2.7.4 Ideal requirements

It is claimed that an ideal depilatory should detach the papilla from the bulb, separate the ORS and epidermis from the underling structure and cause enough cell destruction to the ORS to allow the hair to be freed (Yates, 1968e).

Clearly, an ideal enzyme depilatory should not damage those proteins present in the final product during depilation. An enzyme depilatory should be cheap and stable and it should not have an adverse effect on subsequent skin processing.

2.7.5 Enzyme depilation mechanism – Summary

The mechanism of enzyme depilation appears to involve three critical events; i) the movement of enzymes to target sites; ii) the destruction of certain adhesion molecules in the dermal-epidermal junction; and iii) some degree of destruction of the cellular material of the outer root sheath. The following model for enzyme depilation seems quite plausible (Brady *et al.*, 1989b). It describes that, the depilation load initially falls quickly during enzyme depilation then slows, which fits neatly into the three step mechanism. Initially bonds which are readily accessible and easily cleaved by the given enzyme are broken. Once all these are gone, bonds that are less accessible and more difficult to break by the specific enzyme activity are broken at a slower rate until enough bonds are broken to allow wool pull. An alternative explanation for this pattern is the fast rate of separation between the epidermis/ORS and the underlying structure followed by the relatively slower breakdown of sufficient cells in the ORS reaching a depilation load low enough to allow pulling.

It is unclear from the literature whether the separation of the ORS/epidermis from the underlying structure is a requirement of depilation but it seems that this phenomenon is uniformly present during early stages of enzyme depilation.

Overall, the literature indicates that diffusion of the enzyme to the important proteolytic sites is of initial importance. Once penetrated broad spectrum proteases are good at achieving the destruction required for wool loosening. The various proteins located around the cells of the epidermis and of the basal layer appear to be the general target proteins. This region which includes the basal layer and epidermal extracellular space could be called the enzyme depilatory active region. While targeting specific proteins in this region would be one method of identifying a suitable enzyme for a commercial process, because of their large numbers, the identification of the proteins that remain intact in the finished product would be a more logical approach. This would allow choices to be made about enzymes and processes that were less likely to harm them.

2.8 Protease specificity and inhibition

Since the beginning of fellmongering when skins were depilated through the use of an uncontrolled enzymatic process called “sweating”, there have been problems associated with adverse effects to the quality of the leather. The removal of wool was always accompanied by damage to the valuable part of the skin (grain surface) (Passman, 1997). The problems of damage to the collagen during depilation were largely solved with the advent of lime and sodium sulfide-assisted depilation, due to the high pH and the reducing conditions that inhibited bacterial growth (Pelczar *et al.*, 1993). However, with any enzymatic method of depilation the problem of damage to the valuable skin substances by enzymes remains.

Specific protease inhibitors are available that can be used to limit proteolysis in both industrial and laboratory scales (North, 1989). Specificity of inhibition is related to the mechanism of proteolysis and the specificity of the protease. Thus it is possible to use protease inhibitors to determine which classes of protease are active during the sweating process or during the best practice enzyme depilation. This could be a way of

determining which proteases are beneficial and which are destructive in the enzyme depilation process.

Thus it might be possible to use specific protease inhibitors to prevent the undesirable protease activities during enzyme depilation whilst leaving more desirable activities intact.

As noted in section 2.7.4 enzyme depilation can be achieved using broad-spectrum proteolysis, which results in unwanted damage to collagens particularly at the grain surface. In addition, some prevention of unwanted proteolysis has been achieved during the depilation process by enzyme inhibition through the addition of a known metallo-protease inhibitor - ethylenediaminetetraacetic acid (EDTA) (Allsop T.F. 2003 unpublished work), although overall depilation was not complete resulting in an unacceptable product.

A focussed investigation on inhibition of unwanted proteolysis during enzyme depilation has never been carried out. It is for these reasons that an overview of protease classes and their inhibitors is given in more detail below:

Protease inhibition can be classed into two groups: endogenous protease inhibitors and exogenous protease inhibitors. Endogenous protease inhibitors are those which are present within the animal's metabolic pathways, while exogenous protease inhibitors are those synthetic and microbiological, plant, or animal sourced inhibitors that act on proteases from different sources (Katunuma *et al.*, 1983). For the purpose of this review proteases and protease inhibitors mentioned can be assumed to be exogenous unless stated otherwise.

2.8.1 Proteases and inhibition: By protease class

Proteases can be grouped into four classes serine, cysteine, aspartic acid, and metalloproteases. Their defining characteristics are their catalytic site, activity, mechanism and sensitivities to inhibitors (Barrett A.J., 2001).

Each class has members that are able to hydrolyse extracellular matrix proteins. The actions of some glycosidases are also likely to impact on the degradation of extracellular proteins by removing protective glycosaminoglycans from the extracellular matrix (Sternlicht & Werb, 1999).

2.8.1.1 Serine Proteases

Serine proteases are characterised by a specific triad of amino acids at the catalytic site that has a similar architecture in all known members of the family (Kraut, 1977). They have a specific pH optima, that can vary between acidic (pH 4) and basic (pH 8.5) depending on the enzyme.

It is interesting to note here that plasmin/plasminogen, which is involved in the hydrolysis of fibrin and other cell adhesion molecules, is involved in the movement of neuron cells across a surface. (Leprince *et al.*, 1990). Plasmin/plasminogen may therefore be a promising candidate for the destruction of the cell adhesion molecules in the depilation process, and leave desirable collagens undamaged.

The serine protease trypsin is commonly used in bating, when it helps remove non-collagenous proteins from the skin matrix. Although it has not been found to have any distinct depilation activity, the extracellular matrix activities of this serine protease may augment the depilation activities of other enzymes (Yates, 1972). It should also be noted, however, that industrial bates (containing trypsin) can cause damage to the grain enamel of leather when applied to the skin during processing (Cooper & DasGupta, 2002).

Using relatively insensitive analytical techniques, it has been shown that only a small proportion of the skin proteins remain after processing to the “pickle” stage. Retained materials include various types of collagen and a small quantity of proteoglycans (Alexander *et al.*, 1986), (Deb Choudhury *et al.*, 2006). Therefore the damage caused by exposure to the “bating” process has been attributed to the tryptic destruction of collagens. It is possible however, that the damage observed could be due to other contaminating proteases such as chymotrypsin, present in the industrial bate product.

There are many well characterised serine protease inhibitors. Serine proteases are inhibited by proteins such as the serpins or chemicals such as phenylmethylsulfonyl fluoride (PMSF) and can be either irreversible or reversible. Irreversible inhibitors include: organophosphates such as diisopropylphosphofluoridate (DFP), Sulfonyl fluorides such as phenylmethylsulfonyl fluoride (PMSF), and coumarins such as 3, 4 dichloroisocoumarin (3, 4, DCI) and many others.

Reversible inhibitors include: extended boronic acid peptides; protein inhibitors such as avian ovo-mucoids; kunitz type inhibitors; soybean trypsin inhibitor; and serpins (Salvesen & Nagase, 1989).

2.8.1.2 Cysteine Proteases

These proteases have acidic pH optima but still show some activity at neutral pH (Sternlicht & Werb, 1999). The enzymes are commonly found in lysosomes and probably contribute to depilation during "acetic acid depilation" (Carrie *et al.*, 1956). They are characterised by having an essential cysteine residue at the catalytic site, and can be inhibited by cysteine protease specific inhibitors such as E-64 (Sternlicht & Werb, 1999). Their catalytic mechanism is based on a covalent mechanism that is similar to that of the serine proteases.

There are three categories of cysteine protease inhibitors: peptide diazomethanes, peptide epoxides and natural protein inhibitors (the cystatins). Peptide diazomethanes are oligopeptides with a diazomethyl group attached at the carboxylate terminal. They are irreversible inhibitors and are quite cysteine protease specific (Salvesen & Nagase, 1989). Peptide epoxides include E-64 and its derivatives all of which are also irreversible. Finally the cystatins are a group of proteins which form a tight reversible bond to the active site of cysteine proteases.

2.8.1.3 Aspartic Acid Proteases

Aspartic proteases have an essential asparagine residue at the active site, and are able to hydrolyse a broad range of substrates. They are inhibited by peptide based inhibitors such as pepstatin (Yamato, 1995). Aspartic acid proteases do not normally attack the extracellular matrix proteins (Sternlicht & Werb, 1999). However, the lysosomal protease cathepsin D is an aspartic protease and therefore may be involved in "acetic

acid" depilation (Faust *et al.*, 1985). These enzymes appear to be mainly associated with the breakdown of food by gastric juices, or the wholesale destruction of proteins by lysosomal enzymes (Blundell *et al.*, 1998).

Aspartic proteases can be divided into two groups, retroviral or pepsin like (Pitts *et al.*, 1995). They are active and stable in acid to neutral solutions and thus may be incorporated into "acid bates" (Yongquan, 2001). They have an overall broad specificity but prefer to attack peptide bonds between hydrophobic amino acid residues (Andreeva *et al.*, 1995).

The classic inhibitor for aspartic proteases is pepstatin A (Salvesen & Nagase, 1989). However, as all known aspartic acid proteases (apart from rennin) are active below pH 7 (Salvesen & Nagase, 1989) and, as was noted earlier, the best depilation so far observed is carried out at alkaline pH, it is therefore unlikely that aspartic acid proteases would be useful during an alkaline enzyme depilation. It is possible however that residual activity of endogenous aspartic acid proteases not destroyed during alkaline depilation may be present during the pickling process and may therefore be related to subsequent skin damage.

2.8.1.4 Metallo-proteases

As the name suggests the defining characteristic of metallo-proteases is that they require a metal ion such as Zn^{2+} or Ca^{2+} to bind at the active site for activity. They can therefore be inhibited by the addition of metal chelators such as EDTA (Ethylenediaminetetraacetic acid) (Salvesen & Nagase, 1989). Since EDTA has been found to reduce proteolytic damage to skins, (Allsop T.F. unpublished work) it is possible that metallo-proteases are one of the causative agents for damage.

The metallo-proteases which are known to degrade extracellular proteins are varied and can be divided into 5 subclasses (Sternlicht & Werb, 1999). These include the subclasses: Metzincins and gluzincins.

2.8.1.4.1 Metzincins

The metzincins are a subclass of proteases of particular interest in terms of the extracellular matrix proteins, and can be categorised into four separate families: The

ADAMS comprising the amalysin, astacin and serralysin families, and the matrix metallo-proteases (MMP's). Their amino acid sequences are characterised by a zinc binding motif, and zinc is an essential cofactor. The family to which each metzincin belongs is determined by their amino acid sequence(Stocker *et al.*, 1995). MMPs are of particular interest as they have been named as being important in the *in vivo* remodelling of the basement membrane proteins such as collagen, fibronectin, and laminin (Bode, 2002).

2.8.1.5 Non-specific protease inhibitors

2.8.1.5.1 Multi-class protease inhibitors

Some protease inhibitors are not limited to inhibiting only one class of protease. These include peptide chloromethyl ketones such as tosyl-lysine chloromethyl ketone (TLCK) tosylamido-2-phenyl-ethylchloromethylketone (TPCK), and α-macroglobulins. The latter forms a complex with the protease but does not specifically interact with the catalytic site; Instead, the substrate is hindered from reaching the catalytic site by the binding of a specific sequence of α-macroglobulin (Salvesen & Nagase, 1989).

The breakdown of the cartilage matrix by hydrolytic enzymes released from dead cells is inhibited by either pepstatin or the anti serum to cathepsin D (Dingle, 1976). It may therefore be possible to limit the damage occurring to collagen during enzyme depilation if the damage is the result of endogenous cathepsin D or related proteases also susecptable to these. While inhibitors such as TLCK, TPCK, EACA, PPP, aurolthiomelate, penicillamine and o-phenanthroline have failed to control the reabsorbtion of connective tissue by live cells they may prove to provide effective protection against undesirable proteolytic activity in enzyme depilation. Unfortunately the cost of these laboratory chemicals is likely to rule them out of industrial processing unless their cost effective use can be shown.

2.8.1.5.2 Metal chelating substances

Some proteases require metal ions to stabilise their tertiary structure in contrast to being part of the catalytic machinery (Bode, 2002). The effective removal of these ions through the addition of a metal chelator such as EDTA will therefore also inhibit proteolytic activity (Salvesen & Nagase, 1989).

2.8.1.5.3 Indirect reduction in unwanted protease activity

It may be possible to direct selective proteolysis in enzyme depilation by controlling the susceptibility of collagens and other desirable proteins to protease attack during the depilation process. Conversely, it may be possible to attenuate the activity of specific proteases so they cause less skin damage. This could be achieved via the addition of salts to change the ionic strength of the environment, alteration of pH, the use of reducing agents, processing at low temperatures, the addition of chaotrophs such as urea, dimethylsulfoxide (DMSO) or ionic detergents such as sodium dodecyl sulfate (SDS) (North, 1989).

2.8.2 Proteases and inhibition: By protease substrate

2.8.2.1 General extracellular matrix specific exogenous proteases

Commercial production of proteases for industry, which may require many tonnes annually, has resulted in a range of enzymes that have been purified from animal or plant origin, often as a by-product. When enzymes from more unusual sources are required on an industrial scale they are usually recombinant enzymes produced by fermentation methods. Proteases from exogenous sources tend to be characterised in terms of overall efficiency against test substrates such as casein. When activity against extracellular matrix proteins are characterised the collagenase activity is usually the criterion used.

There has been considerable work investigating the impact of endogenous proteases in medical pathology resulting in a wealth of knowledge about the susceptibility of many ECM proteins to specific proteases. A table of known endogenous proteases and their substrate specificities is given in Table 4.

Table 4: Endogenous enzymes capable of digesting extracellular matrix proteins

Name	Type	EC number	Substrates
Cathepsin B	Cysteine	3.4.22.1	Proteoglycans Fibronectin Non-helical Collagen I,II,III,IV
Cathepsin K	Cysteine	3.4.22.38	Collagen I Elastin Osteonectin
Cathepsin L	Cysteine	3.4.22.15	Elastin Collagen
Name	Type	EC number	Substrates
Cathepsin S	Cysteine	3.4.22.27	Elastin Collagen
Name	Type	EC number	Substrates
MMP-1 Collagenase 1	Matrix metallo protease (MMP)	3.4.24.7	Collagens I, II, III, VII, X, gelatines entactin, link protein, aggrecan, tenascin
MMP-2 Gelatinase A	MMP	3.4.24.24	Collagens I, IV, V, VII, X, elastin, fibronectin, gelatines, link protein, aggrecan, vitronectin
MMP-3 stromelysin-1	MMP	3.4.24.17	Proteoglycans, laminin, Collagens I, II, III, IV, V, IX, X, XI elastin, fibronectin, gelatines, link protein, entactin, fibrin, tenascin, vitronectin
MMP-7 matrilysin	MMP	3.4.24.23	Proteoglycans, laminin, gelatines, collagen IV, entactin, fibronectin, link protein, vitronectin, elastin, tenascin
MMP-8 collagenase-2	MMP	3.4.24.34	Collagens I, II, III, gelatines, aggrecan
MMP-9 gelatinase B	MMP	3.4.24.35	Gelatine, collagens IV, V, XI, elastin, aggrecan, link protein, vitronectin, galectin-3
MMP-10 stromelysin-2	MMP	3.4.24.22	Proteoglycans, laminin, gelatines, elastin, collagen I, II, III, IV, V, IX, fibronectin, link protein
MMP-11 stromelysin-3	MMP	3.4.24.B3	Laminin, fibronectin, aggrecan

MMP-12 metalloelastase	MMP	3.4.24.65	Elastin, fibrinogen, fibronectin, laminin, entactin, collagen IV, proteoglycans, myelin basic protein,
MMP-13 collagenase -3	MMP	Not assigned	Collagens I, II, III, gelatines, aggrecan
MMP-14 MT1-MMP	MMP	3.4.24	Collagens I, II, III, gelatines, fibronectin, laminin, vitronectin, proteoglycans
MMP-15 MT2-MMP	MMP	3.4.24	Collagens I, II, III
MMP-16 MT3-MMP	MMP	3.4.24	Collagens I, II, III
Name	Type	EC number	Substrates
MMP-17 MT4-MMP	MMP	3.4.24	Collagens I, II, III
MMP-18 collagenase-4	MMP	Not assigned	Collagen I, gelatines
MMP-19	MMP	Not assigned	Similarities to stromelysins
MMP-20 enamelysin	MMP	3.4.24	gelatine
Name	Type	EC number	Substrates
MMP-21	MMP	Not assigned	Unknown
MMP-22	MMP	Not assigned	Unknown
MMP-23	MMP	Not assigned	Unknown
MMP-24	MMP	Not assigned	Fibronectin, not collagen I, not Laminin
Meprin	MMP	3.4.24.18	Pro-collagens, collagen IV, laminin, fibronectin, gelatine,
plasmin	serine	3.4.21.7	Fibronectin, vitronectin, laminin, proteoglycans

From (Sternlicht & Werb, 1999), (sigmaaldrich, 2006), (Bankus & Bond, 2001)

Proteases that may be of interest to enzymatic depilation processes (multiple ECM activity with no stated collagenase activity (except collagen IV)) are shaded grey.
An interesting class of endogenous protease which appears to have no activity against any form of collagen is MMP-11. While this does not preclude it from causing damage to the skin during depilation, it is therefore a target for further investigation.

2.8.2.2 Collagenase

Due to its nature as both a structural protein and as a potential food source, enzymes that specifically breakdown collagen are common. “Collagenases” are produced by bacteria as a way of breaking down the collagen into peptides that can be further broken down by peptidases and absorbed by the gut. In animals they are part of a catabolic pathway required for reshaping structural components. A “true collagenase” can be

defined as an enzyme that degrades only collagen in an un-denatured, triple, helical, form (Peterkofsky, 1982) rather than the globular portion of some collagens or denatured collagen. Additionally, although some bacterial collagenases are only able to degrade collagen in its un-denatured form, they can therefore degrade gelatine.

Leather is almost completely composed of collagen and destruction of the collagen matrix has a major impact on the quality of the leather. If micro-organisms are allowed to proliferate on the skin, "staling" will occur as the collagenases released by these micro-organisms create patches of damaged collagen visible on the surface of the leather as well as damaging the internal structure resulting in "loose" leather. These effects are detrimental to the final product and are therefore costly to the fellmongery industry. Clearly, minimisation or elimination of damage to collagen by collagenase is of prime concern during enzyme depilation.

Certain skin diseases have been associated with collagenases arising from colonisation of the skin by pathogens such as *Streptomyces medurae*. Collagenases are also produced by the pathogens *Clostridium perfringens*, *Clostridium Histolyticum*, and *Pseudomonas aeruginosa* (Mier & Cotton, 1976). Clostridial collagenase has been shown to attack the basement membrane collagen as opposed to the collagen fibre bundles, so care must be taken when describing an enzyme as a collagenase. For example (Burgeson *et al.*, 1990) claimed that both type IV and type VII collagens are hydrolysed by a type IV collagenase (Seltzer *et al* (1989), (Burgeson *et al.*, 1990). This implies that an enzyme designated as having activity to a specific collagen type may also attack other collagen types.

This raises an important point. In addition to the specificity of a proteolytic enzyme's active site, the ability of a protease to breakdown a range of proteins is related to that part of the substrate protein which is susceptible to proteolysis. Evolutionary theory suggests that proteins with related functions have related structures. This protein homology (or similarity) can indeed be found in the proteins of the extracellular matrix, in particular regulating protein-cell interactions (Chothia & Jones, 1997). Certain structures within individual proteins exist that share the same peptide sequence known as epitopes or even domains containing groups of epitopes. These peptide chains can be found in different proteins resulting in proteases, which are highly specific to a given

peptide sequence, having the ability to breakdown different proteins by hydrolysing regions of the same epitope that are present on those different proteins.

As an approximation of the epitope specificities described here the investigation of proteolytic enzymes with selected whole protein specificities was carried out in later sections. It is suggested that future work could be carried out to investigate the relationship between proteins of interest and the specific epitopes susceptible to proteolytic hydrolysis which they contain, resulting in a more difficult but more targeted determination of possible protease or protease mixtures.

2.8.2.2.1 Collagenase inhibitors

The control of the process of catabolism of collagen *in vivo* requires the use of collagenase and collagenase inhibitors (Weiss, 1984). A number of collagenase inhibitors have been identified in tissue. It appears that endogenous collagen inhibitors are often low molecular weight (<50000) proteins, such as β_1-anticollagenase (Weiss, 1984), which are specific to the collagenases located in the tissue in which the inhibitors are found. These inhibitors are unlikely, however, to inhibit bacterial collagenases. Therefore the identification and subsequent biochemical and structural characterisation of the exogenous collagenases is the most likely way of identifying or synthesising inhibitors to control undesirable collagenolytic activity. This possible path of investigation is left for future investigators.

Although endogenous collagenase inhibitors are not the subject of this review the endogenous protease inhibitor for human leukocyte collagenase is of interest (Tschesche & Macartney, 1983). The protease is reversibly inhibited by a small protein about 25kDa, which interestingly inhibits some other non-related collagenases. It might be interesting in the future to trial this inhibitor during enzyme depilation.

2.8.2.3 Keratinase

Keratinases are essentially unknown in mammals. There is a continual turn over of proteins in living tissues but, although enzymes are responsible for the catabolism of many proteins, keratin is an exception (Mier & Cotton, 1976). Keratin, which is the major structural protein in hair, is removed by shedding from the skin of the animal

which is seen by some as another mode of excretion because of the relatively high concentration of heavy metals in hair. Thus only a select few keratinases, capable of breaking down native mature keratin (Mier & Cotton, 1976), have been found, and in only pathogenic organisms such as *Tricophyton schoenleini*, *Tricophyton rubrum*. and *Tricophyton mentagrophytes*. These organism produce keratinises that have pH optima of 8-9 (Mier & Cotton, 1976). The possible use of keratinases as a means of depilation has been discussed in section 2.7.2.1.3.

2.8.2.4 Elastase

A number of enzymes have been found to damage elastin including commercial enzyme products used in skin processing industry such as pyrase (NovoNordisk, Denmark) (Lowe, 1997).

Enzymes that are claimed to have elastase activity include serine proteases such as neutrophil elastase (EC 3.4.21.37), metallo proteases such as MMP's 2,7,9,10,12 (weak MMP 3); and cysteine proteases such as Cathepsin S

2.8.2.5 Glycosidase

The initial degradation of proteoglycans requires removal of the polysaccharide chain from the protein backbone (Mier & Cotton, 1976). The first step in this process may be hydrolysis of the protein backbone. Subsequently the glycosaminoglycan may be broken down by specific enzymes such as the "hyaluronidases" chondroitinase, and sulfatase. All previously discovered hyaluronidases are endo-β-acetylhexosaminidases (Mier & Cotton, 1976) with acidic pH optima (testicular hyaluronidase being the exception). Enzymes capable of directly breaking down dermatan sulfate are unknown implying that de-sulfation has to occur before the GAGs become susceptible to enzymatic depolymerisation (Mier & Cotton, 1976). Studies on rat showed that the natural half lives of hyaluronic acid and dermatan sulfate were 1.7 days and 7 days respectively (Mier & Cotton, 1976) which suggests that there must be enzymes responsible for their catabolism.

2.8.2.6 Minor components of the extracellular matrix proteolysis

The endogenous control of cell-cell and cell-(extracellular matrix) interactions appear to be carried out by a proteolysis cascade system similar to the clotting thrombin cascade. Although the final endogenous proteolytic hydrolysis of the adhesion molecules is carried out by plasmin, the availability of plasmin to certain sites *in vivo* is controlled

through interactions with the precursor cascade (Sternlicht & Werb, 1999). The implication is that hydrolysis of pro-enzymes by an exogenous enzyme may indirectly result in the hydrolysis of extracellular matrix components through the activation of endogenous proteases

2.8.3 Protease specificity and inhibition – Summary

Reports indicate that inhibition of damage causing proteases may be possible through a number of means including the addition of exogenous protease inhibitors specific to the damage causing protease, or by use of endogenous inhibitors specific to the damage causing proteases. These methods are unlikely to be commercially viable due to the high cost and health risks associated with some reagents.

The depilation process appears to require broad specificities in order to break down the material found in the depilation active region and it is possible that inhibition of specific proteases may result in inadequate loosening. An investigation into the use of specific inhibitors for enzyme depilation is merited, but due to the scope of the problem is a project in its own right that will be left to future investigators.

Alternatively the use of non specific protease inhibitors may be a viable method of determining which enzymes are responsible for the damage that occurs naturally in “sweating” and enable depilation conditions to be ascertained that minimise damage to the skin while still allowing depilation to occur.

2.9 Literature review – Conclusions

A review of the relevant literature indicated that enzymatic depilation is a desirable alternative to conventional lime/sulfide based processing and most likely to be achieved through the use of a broad spectrum alkaline stable endo-protease. In order to determine the mechanism of depilation and the problems associated with such a process it is clear that a selection of these enzymes be obtained and characterised.

Significant literature exists describing depilation through the use of enzymes or with the assistance of enzymes. The major focus has been on the ability of a given available protease to achieve depilation and not on the problems associated with enzymatic depilation or the mechanism of the processes involved. Problems are described however, including the retention of some fibres indicating some unacceptable variation in depilation. Varying levels of damage to the skin surface are also reported resulting in unacceptable leather. These two problems are linked in that addressing a reduced level of depilation effectiveness in a poorly depilated region may lead to overcompensation resulting in damage in a region which was easily depilated. While this issue may be simple to solve when applying conventional lime/sulfide depilation, it would therefore be useful if the causes for variation in an enzymatic depilation system could be mapped.

Previous investigations into the mechanism of enzymatic depilation have shown that destruction of the links between the wool fibre and the surrounding skin matrix is the key to enzymatic depilation. Since neither the cause of depilation nor the cause of damage is known at a protein level the educated selection of a protease which can depilate without damage cannot proceed until these are determined. An examination of the literature gave a range of possible proteins that may be involved through their interaction with the proteins and structure of the extracellular matrix. A determination of the proteins which are important to enzymatic depilation and those that must be retained to maintain high quality leather are therefore required.

The literature showed that there is considerable knowledge about proteolysis in terms of types of proteases, their substrates and the means to control their activity. It was found

that it may be possible to manipulate the depilation process through modification of the depilation conditions or selection of the right kind of protease resulting in a successful depilation without damage to the skin.

Overall enzyme depilation, which appears to be a sound research direction, is limited by the fact that the described processes do not produce a pelt product that is both acceptable in terms of being wool-free, sulfide-free, and damage-free. No single line of research appeared to offer a direct solution to these problems. In order to develop an enzyme based depilation process it is therefore necessary to do the following:

- Determine the nature and basis of enzyme caused damage to the skin so that the issue of an acceptably damaged product can be addressed. This work is attended to in chapter 3.
- Determine both the physical and chemical mechanism of enzymatic depilation so that the problem of variable and incomplete depilation can be addressed. This work is attended to in chapter 4.
- Identify the important components in the skin required for prevention of damage in the finished leather. This work is attended to in chapter 5.
- Assess the best options for the development of a successful enzyme depilation based on the nature of enzyme depilation damage and the enzyme depilation mechanism. This work is attended to in chapter 6.

Experimental characterisation of depilation

3.1 Introduction

The aim of this chapter is to carry out enzymatic depilation, to characterise the general attributes of the process, and to determine the nature of the faults with the enzymatic depilation process. A comprehensive review of the literature showed that the uptake of this process by industry has been slow, despite there being a large number of published reports on enzymatic depilation. Attempts at applying ostensibly successful enzyme depilation methods previously described in the literature, have been unsuccessful in New Zealand. Since, in general, public reports in the literature tend to describe benefits of the enzymatic depilation process and not its faults a detailed characterisation of those faults is long overdue. Without detailed knowledge of what is wrong with enzymatic depilation, a successful process is unlikely to be developed. For these reasons a number of proteolytic enzymes was trialled and the effect of them on the depilation of New Zealand lambskins assessed. The specific faults were then described so that they could be investigated in later chapters.

3.2 Enzyme depilation

In order to begin a detailed examination of the enzyme depilation process a series of preliminary experiments was carried out. The purpose of these experiments was to confirm literature findings and to prepare a starting point for the analysis of the mechanism of enzyme depilation. As described in the literature review, the most effective enzymes to effect depilation, were broad spectrum endo proteases, particularly those that were active in alkaline conditions. These then were the features of the initial enzyme selection.

3.2.1 Initial experiments

Fresh Romney cross lambskins (green skins) were obtained throughout the season (from lambs aged 2 to 12 months) from a co-operating meat plant and pre-fleshed immediately prior to use in the depilation experiments.

The depilation process is complex, requiring multiple activities that are largely unmapped. There is no specific bioassay for depilation that can be used to standardise enzyme depilatory formulations. Assay techniques have been reported that have been used to screen for unwanted activity against collagen (Yates, 1968d), but these are not appropriate for screening depilation activity. Enzymes were therefore applied directly to skins and the depilation assessed manually. Solutions of enzyme preparations 10% (v/v) were used for an initial evaluation of their depilation ability. The enzymes selected for screening are listed in Table 5. As a basis for selection, a range of proteolytic enzymes trialled that had stability to alkaline conditions and possessed a broad proteolytic specificity.

3.2.1.1 Table of enzymes sourced for this work

Table 5: Enzymes selected for screening

Name	Type	Supplier
Promod 278P	Mixture	International Sales and Marketing N.Z.
Protease 6L	Alkaline Bacterial	Genencor International
Protease 200	Neutral Bacterial	Genencor International
Batinase Neutral	Neutral Bacterial	Genencor International
Bromelain	Plant	Genencor International
Purafect	Alkaline Bacterial	Genencor International

3.2.1.2 Enzyme application method

For enzyme testing, a biocide (or bacteriostat) was required to ensure that the skins were not affected by bacterial colonisation during the course of the depilation (Yates, 1964). Recent work has shown that the use of dithiocarbamate based bacteriostats provide good protection on fresh skins without affecting the activity of a range of proteolytic enzymes (Cooper & Lowe, 1997).

Previous work had also shown that the use of alkaline pH and particularly, alkaline stable proteases provides additional depilatory effectiveness during enzymatic depilation (Yates, 1968b), (Berla Thangam & Nagarajan, 2001). It had also been reported that an alkaline treatment was required for skins that were to be dewooled using proteases (Brady *et al.*, 1989b) in order that the process yields skins that have similar properties to conventionally processed skins. For these reasons alkaline operating conditions were chosen and generated using a readily available and relatively inexpensive sodium carbonate/bicarbonate buffer system at pH 10.5 with the intention that the optimal pH and buffer system be determined during the course of the work.

The exact enzyme formulation used in the depilation trials is given in Table 6 below:-

Table 6: Enzyme application formulation

Reagent	Purpose	Supplier
0.2% (v/v) Busan 85 (potassium dimethyldithiocarbamate)	Bacteriostat	Buckmans, Australia
0.5% Teric BL8	Surfactant/ wetting agent	Huntsman, Australia
0.5 M sodium carbonate/sodium bicarbonate buffer at pH 10.5	Alkaline buffer	BDH Chemicals Ltd (Poole, England)
10% w/v test enzyme	Depilatory enzyme	Variable

Either whole skins, matched halves or pieces (5 × 5 cm) cut from the skin at the official sampling position ((IUP 2, 2000)) were used to examine the depilation process. The skin being used was fully immersed in the enzyme solution and then held in an immersed state for 30 minutes.

After a wet holding period of 30 minutes the pieces were “hydro extracted” (see glossary) to recover the residual enzyme solution, and held overnight (20 hrs) at 20°C. The wool was then removed by hand, the ease of removal subjectively assessed, and the quantity of both removed and residual wool weighed. To hand dewool, pressure was applied to the wool by the thumb and drawn across the skin. The amount of pressure applied was increased until depilation occurred or until grain cracking could be observed at which point the depilation was discontinued.

3.2.1.3 Depilation results

The results from the initial enzyme screening trials are shown in Table 7 below.

Table 7: Enzyme depilation results

Enzyme	Ease of depilation	% Wool removed
Promod 278P	Easy	99%
Protease 6L	Easy	100%
Protease 200	Very Easy	95%
Batinase Neutral	Very easy	99%
Bromelain	Medium	50%
Purafect 4000L	Very easy	100%

The only enzyme formulation that did not achieve excellent wool removal under these conditions was Bromelain. The other enzymes left residual wool remaining only on the edges of the test pieces. This is important, because residual wool at the edges of pelts is much less of a practical concern than residual wool in the main panel of the skin. It is possible that liquid diffusion out of the edge, or the inability to get a good purchase on the wool during removal at the edge caused this.

3.2.1.4 Wool product

The literature reports on the impact of enzymes on wool have been conflicting. Some authors state that enzyme depilation could be achieved at the expense of proteolytic destruction of the wool (Brady *et al.*, 1987), whilst others report that wool was mostly resistant to enzyme degradation (Addy, 2000). The different reports probably relate to different expectations about the mechanism of wool loosening using enzymes. These relate to either the destruction of the proteins which hold the wool in place, or destruction of the fibre itself. The effects of immersion application of enzyme depilation formulations to green skins were therefore investigated in order to determine the type and extent of damage to the wool itself.

3.2.1.4.1 Methodology

The wool recovered was assessed using tests based on the measurement of its solubility in both alkali and acid. Alkaline solubility was chosen because it is a standard method to

assess general damage to wool (Jackways, 1987). For the most part when wool has been damaged, the fraction of a given weight of wool that is dissolved using a standard alkaline treatment protocol increases.

However, when wool is subjected to alkaline conditions during enzyme processing, it is possible that a portion of the sample that is more susceptible to alkaline dissolution will already have been removed from that sample prior to testing. The sample will therefore appear to be less alkali soluble in the test and damage assessed by the alkali solubility test will therefore be underestimated. This possibility was considered during analysis of the results.

During the enzyme depilation process, the wool was exposed to a pH 10.5 buffered solution, which may result in spurious solubility data from the alkali solubility test. Therefore, an acid solubility test was also used as an alternative method of determining possible wool damage that was not compromised by the effect on alkali solubility as discussed above.

3.2.1.4.2 Alkali solubility

The methodology of (Jackways, 1987)was used. Briefly, samples of wool were degreased by extraction with light petroleum (boiling point 40-60°C) for 1 hour (6 cycles per hour) in a Soxhlet apparatus. The extracted samples were cut to 1 cm lengths and allowed to equilibrate overnight at 20°C / 65% relative humidity (r.h.). This wool was sub-sampled and the moisture content determined by drying at 105°C. Weighed samples (~1g) of the degreased/equilibrated wool were placed in to 100ml of 0.1M NaOH for 1 hour at 66°C in a shaking water bath. The wool was then washed six times in distilled water, two times in 1%v/v acetic acid, and six times again in distilled water (60 mL each). The retained wool was dried at 105°C and the solubility calculated as the % difference between the initial sample weight (recalculated as a dry weight) and final dry weight of the remaining wool.

3.2.1.4.3 Acid solubility

Degreased wool was prepared as described in 3.2.1.4.2. Samples of the degreased/equilibrated wool (0.5g) were placed in to 50ml of 4M HCl for 1 hour at 66°C in a shaking water bath. The wool was then washed six times in distilled water,

twice in 0.1M sodium bicarbonate solution, and six times in distilled water (60 mL each). The retained wool was dried at 105°C and the solubility calculated as the % difference between the initial sample weight (recalculated as a dry weight) and final dry weight of the remaining wool.

3.2.1.5 Results from the solubility trials

After rinsing in 45°C water all wool samples from the depilation trials were visually the same. The wool recovered from the Bromelain formulation treatment was initially stained due to the colour of the formulation but this colour quickly washed out to produce wool that was clean and indistinguishable from the other samples.

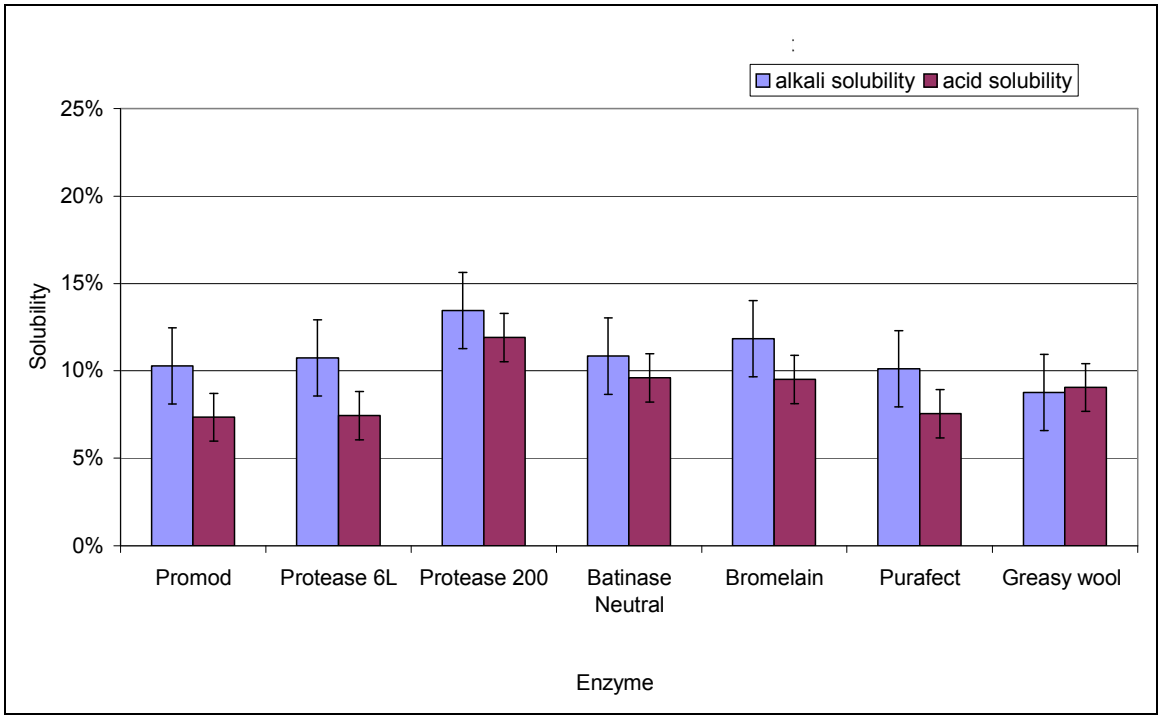


Figure 17: Effect of the enzyme depilation processes on wool, a higher score indicates wool that is more susceptible to the solubility test and hence may be damaged.

The wool product from the alternative enzymes trialled appeared similar to that produced using Purafect as shown in Figure 17. Protease 200 was significantly higher than the results for the greasy wool control indicating that the use of this enzyme may result in some damage to the wool product. The application of the remaining enzymes did not result in significant changes in comparison to the control. Shorn greasy wool was tested to represent the bulk of the wool produced from the conventional process not

including the wool bulb which is destroyed during conventional processing. Shorn greasy wool was found to have alkali and acid solubility results of around 10%.

The results shown in Figure 17 confirm that wool from most of the enzyme depilation treatments had alkali and acid solubility properties that were not significantly different to the bulk of the body wool produced during conventional slipe wool production. The acid solubility results were consistently lower than the alkaline solubility results showing that the alkaline treatment during depilation did not remove alkaline susceptible material from the wool. It can therefore be concluded that acid solubility testing is not necessary when testing the effect of an alkaline enzyme depilation process.

Conventional slipe wool also carries the risk of sulfide paint contamination of the wool at the fringes. This fringe wool is generally separated from the bulk body wool recovered at wool pulling as sulfide causes severe damage to wool. In comparison, wool produced by the enzyme immersion method showed no damage associated with either contamination of fringe wool or with the presence of damaging sulfide on the wool root. The enzyme immersion process followed by removal of the excess enzyme liquor was therefore found to have minimal impact on the wool resulting in no significant damage.

3.2.1.6 SEM (Scanning electron microscopy) of enzyme treated wool

Samples of wool were also examined using Scanning electron microscopy to determine any possible detrimental changes to the surface structure that were not assessed using the solubility tests.

Two wool samples were prepared from the same skin piece. The samples were treated as follows: A negative control sample was prepared by taking some raw wool and washing it with warm water (35°C for 2 minutes). A test sample was prepared by taking a sample of wool from the same greasy wool stock and holding it in wet contact with enzyme immersion solution for one hour (Purafect 4000L, Genencor, Zymus: New Zealand), squeezed of free liquor by hand then held for 16 hours at 20 °C, washed with warm water (35°C, for 2 minutes), then washed with a neutralising detergent solution (20°C for 2 minutes).

The enzyme buffer was prepared as described in Table 6: i.e. 0.5 M sodium carbonate/sodium bicarbonate solution at pH 10.5, 10% w/v Purafect 4000L, 0.5% Teric BL8, 0.2% Busan 85.

The neutralising detergent was made with the following formulation: 3 g/l Tetrapol LTN (surfactant (nonyl phenyl ethoxylate), Shamrock, New Zealand), 1 g/l acetic acid (Lab-scan Asia co. Ltd, Thailand)

The scanning electron microscope images are illustrated below:

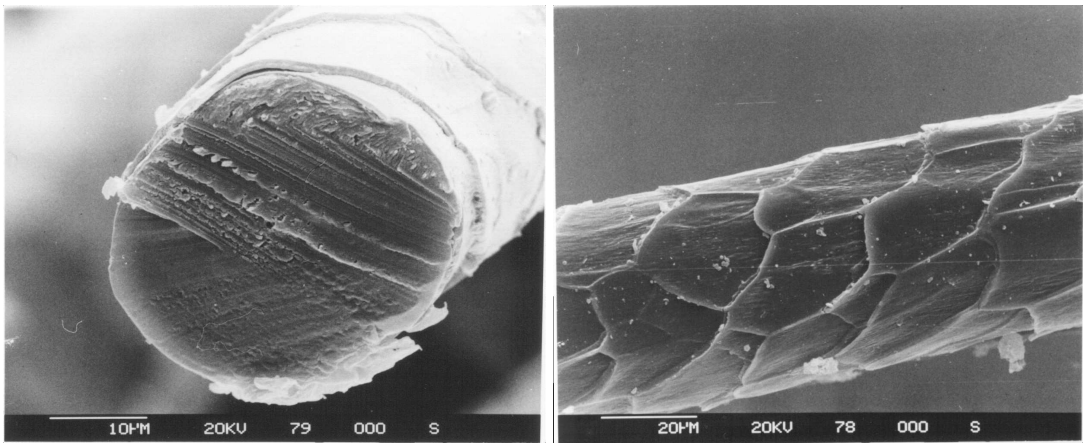


Figure 18: SEM image of greasy wool control, note the cuticle of the wool fibre is visible.

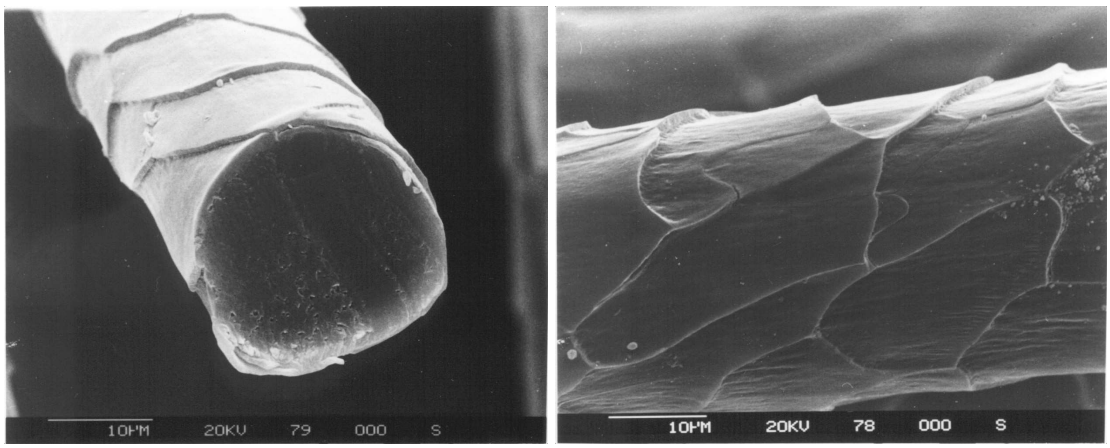


Figure 19: SEM of enzyme treated wool sample, note the defined cuticle indicating that the wool fibre is not damaged by immersion in a buffered enzyme depilation solution after a wash in a neutralising scour.

It is worth noting that washing was carried out to remove the buffer salts. Prolonged exposure to the buffer solution after wool removal from the sheep prior to scouring

would not be recommended. Nevertheless the results show that immersion in a buffered enzyme depilatory solution does not have a detrimental effect on the wool fibre. After the wool was removed from the skin and washed scanning electron microscopy showed that the immersion depilation treated wool fibres were not visibly altered compared to those not treated.

3.2.1.7 Grain surface

The most valuable part of the skin is the grain surface because this is the part of the skin that is visible in the final product, and thus its appearance and integrity is very important. The location of the grain enamel within skin is described in section 2.5. Damage from scars or disease can reduce the value of the skin to the point when processing it into leather becomes uneconomic. Damage from bacterial staling can cause the grain surface to become "dull". The grain surface has a layer known as the enamel, which is a smooth surface at the very top layer of the leather. When damaged, the enamel layer no longer appears smooth and shiny, but becomes scuffed revealing fibrous lower layers (Felicjaniak, 1985). A good grain surface has a smooth regular appearance with no scuffing and regular shaped follicle mouths. An example of good quality grain surface produced from conventional processing is given in Figure 20.

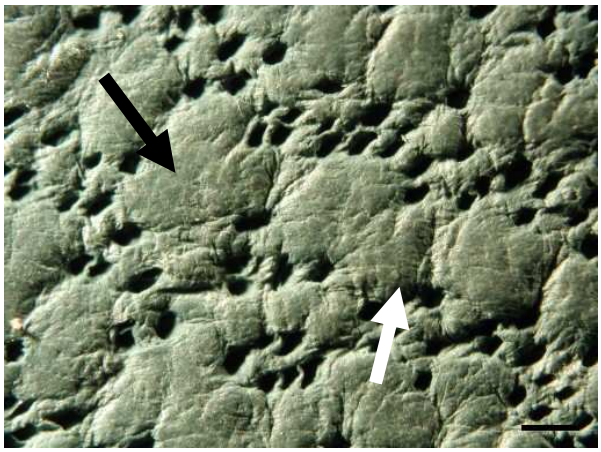


Figure 20: Optical microscopy of grain surface of pelts processed through to crust leather using the conventional lime/sulfide based process. Note the regular appearance of the follicle mouths and smoothness of the interfollicular pads. (The bar is 500 um) (a black arrow indicates an interfollicular pad (hill), a white arrow indicates the valley between two interfollicular pads)

The characteristics of the grain surface of enzyme depilated material are seen in Figure 21 which shows the grain surface appearance of the tanned leather samples from skins depilated with various enzyme formulations.

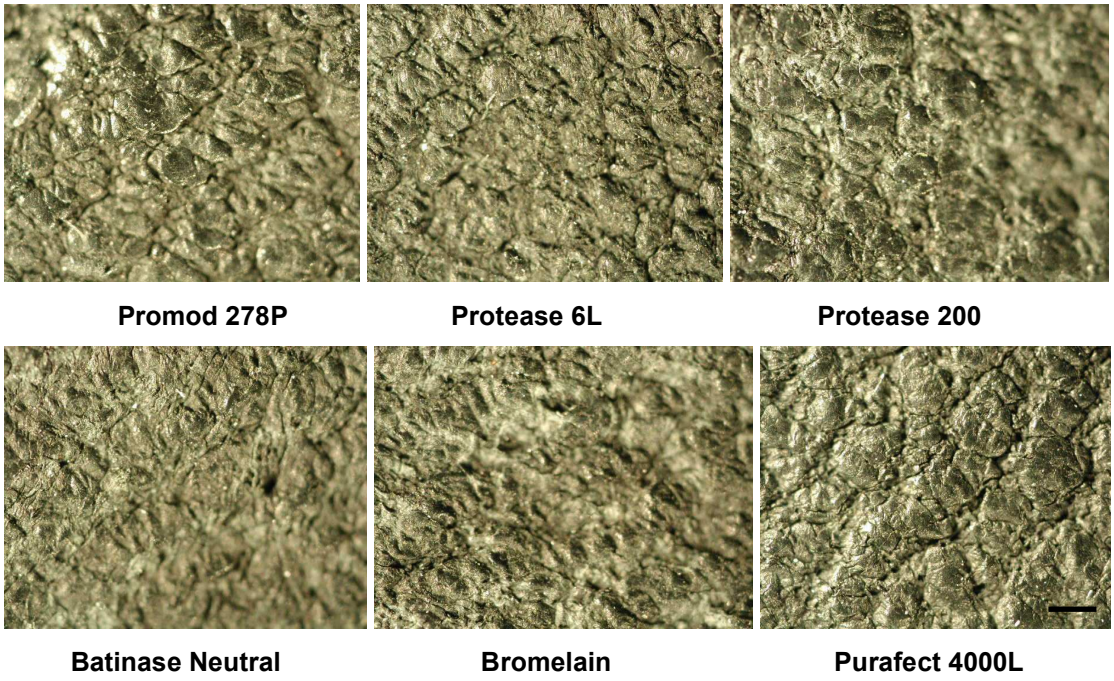


Figure 21: Optical microscopy images of the effect of enzyme process on grain surface (bar is 500 um, all images are at the same magnification).

Both the valleys between inter-follicular pads and the inter-follicular pad surface were examined in this trial. The appearance of the grain surface for each of the enzymes tested is summarised in Table 8

Table 8: Grain surface appearance of tanned skins following enzymatic treatments.

Enzyme	Appearance	
	Follicular pads	Between pads
Promod 278P	Bright	Light scuffing
Protease 6L	Bright	Bright
Protease 200	Bright	Medium scuffing
Batinase Neutral	Medium scuffing	Heavy scuffing
Bromelain	Medium scuffing	Heavy scuffing
Purafect 4000L	Bright	Bright

Most enzyme formulations produced bright, undamaged follicular pads but all the enzymes caused varying amounts of damage to the valleys between the pads. The enzyme formulations that caused least grain layer damage were Purafect 4000L and

Protease 6L, although neither of these would be acceptable in comparison to the conventionally processed material.

3.2.2 Selected enzyme purification

Due to the low purity of the commercial enzymes used, an attempt to purify them was carried out to remove any enzyme activity not required for dewooling but which may be contributing to the damage to the grain structures previously observed.

A selection of methods for separating enzyme components from the commercial enzymes were initially trialled and found to be inadequate. Specifically: Size exclusion gel filtration (SEGF) was trialled which separates proteins on a molecular size basis with the larger molecules being eluted first from the column. Additionally native polyacrylamide gel electrophoresis (Native PAGE) was trialled which separates on the basis of charge and molecular size. These two methods were chosen because of the relative ease of use and availability. Other fractionation techniques such as preferential precipitation using ammonium sulfate, or isoelectric focussing could also have been used.

The column trialled for size exclusion gel filtration was found to be inadequate to generate separated enzyme samples large enough to carry out depilation with. High pH (8.8) native PAGE gels did not separate the enzymes into discernable bands and so was not used (Results not shown).

As an alternative to size exclusion gel filtration separation and the high pH native PAGE separation a low pH native PAGE technique was investigated as a method for isolating individual enzymes from the crude enzyme products.

The crude enzymes can be separated on a polyacrylamide gel without a deactivating sample preparation and no sodium dodecyl sulfate (SDS). This is known as native PAGE or non-denaturing PAGE. The enzyme fractionation results from a separation due to protein charge/size ratio instead of size alone. Different separations occur depending on the pH of the buffer used in the gel. Some enzymes are not separated from their crude formulations because they are not appropriately charged in their native state.

Those that are separated can still be functional enzymes which can be applied to small pieces of skin and the effects examined microscopically using the SACPIC stain described earlier (section 2.2.3.1). This allowed the functional characteristics of enzyme depilation to be observed.

The SACPIC staining technique has been used in the past to determine staling damage and bacterial invasion of skins (Yates, 1968a). The technique was used here to monitor the impact of enzyme reagents on the skin system.

Although high pH native PAGE separation did not successfully separate component proteins from the mixtures (results not shown), low pH native PAGE gels produced a separation of enzymes into different discernable bands and so was used in an attempt to achieve separation of the commercial enzyme reagents.

3.2.2.1 Low pH native PAGE separation method

This system stacks proteins at pH 5 and separates at pH 3.8. The gel is photo polymerised using riboflavin and TEMED catalysts, and the sample buffer contains a tracking dye with a positive charge, in this case methylene blue. The method is based on that previously described (Ornstein & Davis, 1964) the details of which are given below:

Enzymes were first diluted as per Table 9. Then 10 uL samples of diluted enzyme were dissolved in 10 uL sample buffer containing; 20% w/v glycerol, 0.02% w/v methylene blue, 0.0156 M KOH/acetic acid buffer at pH 6.2.

Acrylamide gels (0.75 mm thick, 10% acrylamide) were cast without wells using BioRad Mini-PROTEAN 3 Cell cassettes. The 10% polyacrylamide running gel was prepared with the following formulation; 25% v/v acrylamide/bis (40% solution, BioRad, 161-0148), 2.5 mM riboflavin, 0.1% v/v Temed (BioRad, 161-0800, USA), 0.1 M KOH/acetic acid buffer at pH 4.3.

After 2hrs the photo polymerisation stacking gel was over laid and 10 lanes formed. The stacking gel had the following formulation; 25% v/v acrylamide/bis (40% solution,

BioRad, 161-0148), 2.5 mM riboflavin, 0.1% v/v Temed (BioRad, 161-0800, USA), 0.01575 M KOH/acetic acid buffer at pH 6.2.

In each case the gel solutions were degassed for 2 minutes under a vacuum before addition of the riboflavin and Temed catalysts. (Riboflavin was added in the form of a 20mM riboflavin-5'-phosphate solution). Photo polymerisation was carried out for 2 hours using a 75W incandescent light bulb at 200mm from the gel.

The cast gel was run in a mini-Protean 3 gel electrophoresis Unit from BioRad. 500mL of running buffer was used with the following formulation (Running buffer; 0.08%v/v acetic acid, 31.2g/L β-alanine pH 6). The electrophoresis cell was operated according to the manufacturers instructions with the exception that the electrodes were operated in reverse due to the fact that the pH was low and the proteins were expected to migrate away from the positive terminal under acid pH conditions. 8

Table 9: Dilution factors used in SDS-PAGE based enzyme characterisation

Name	Dilution factor
Batinase ® Neutral	1/10
Bromelain	1/20
Promod 278P	1/10
Protease 200	1/10
Protease 6L	1/10
Purafect 4000L	1/10

The gels were run at 100Volts for about 5 min. When the dye had run into the gel, the voltage was increased to 200V and held at that voltage until the electrophoresis was complete. When the dye had been observed to reach the bottom of the gel, the voltage was turned off and the gels were removed and then stained.

The native PAGE gels were run with an ice pack to maintain a constant temperature and reduce the chance of degradation of the enzymes. After running the gels the large lane was immediately sliced horizontally into strips. The smaller lane was stained to determine which of these excised strips contained bands of protein. Each slice was then macerated in a test tube containing 2 mL buffered dewooling solution (made up of 0.5

M sodium carbonate/ sodium bicarbonate buffer at 10.5 pH, 0.2% (v/v) Busan 85 and 0.5% (v/v) Teric BL8).

1 cm² of sheep skin cut from the backbone was placed into each test tubes. The tubes were then shaken to ensure complete wetting of the skin piece and then held for 16 hours at 20 °C. The 1 cm^2 pieces were subjectively examined for ease of dewooling then placed in buffered formalin solution (see section 8.3.1.2) for sectioning and examination using the SACPIC stain (section 8.3.3).

3.2.2.2 Low pH native PAGE separation of enzymes

In order to isolate enzymes from the crude products that did not separate at pH 8.8, an alternative low pH scheme was trialled.

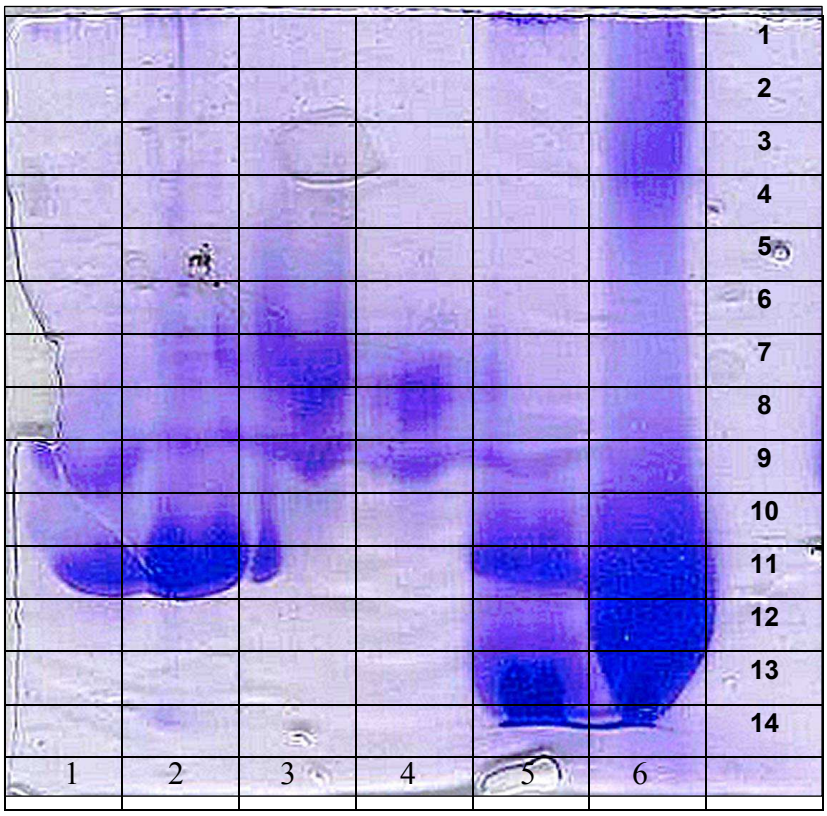


Figure 22: Poly acrylamide electrophoresis separation of enzymes under low pH (4.3) conditions using 10% polyacrylamide concentration. Lanes are numbered along the bottom and equivalent excised gel strip indicated on the right. Lanes were as follows: Batinase ® Neutral, Purafect 4000L, Protease 6L, Protease 200, Promod, Bromelain

It was previously found that a number of proteins were seen to be separated from the raw products under low pH (pH 4.3) conditions. Each raw product was then individually separated using a modified "Ornstein-Davis" method (section 8.4.4):
The results for the gel method are illustrated in Figure 22.

It can be seen that a number of protein fractions could be separated from the raw enzymes using the alternative low pH native PAGE process. When preparing individual enzyme fractions the entire width of the gel was loaded with enzyme and after separation each gel was sliced into 15 strips each 4mm in width. The enzymes in each segment were extracted by maceration of the gel segment in a test tube containing 2ml buffered dewooling solution. The buffer formulation was that described in Table 6 with the exception that the enzyme was supplied in the form of the macerated gel strip:(briefly;0.5 M sodium carbonate/sodium, bicarbonate buffer at 10.5pH, 0.2% (v/v) Busan 85, 0.5% (v/v) Teric BL8).

A piece of sheep skin, 1 cm² cut from the backbone was placed into each of the test tubes. The tubes were then shaken to ensure complete wetting of the skin and then incubated for 17 hours at 20°C. The 1 cm^2 pieces were subjectively examined for ease of dewooling then placed in buffered formalin solution (see section 7.2.1.2) for sectioning and examination using the SACPIC stain. The results are in Table 10.

Table 10: Depilation results for enzymes separated on low pH native PAGE gel

Segment	Batinase ® neutral	Purafect	Protease 6L	Protease 200	Promod	Bromelain
1	**Medium**	Easy	Easy	Easy	**Medium**	**Medium**
2	Difficult	Medium	**V. easy**	Easy	Difficult	Difficult
3	V. difficult	V. easy	Easy	Medium	**Medium**	Medium
4	Difficult	**V. easy**	**Medium**	**Medium**	Medium	**Medium**
5	Difficult	Medium	Medium	Medium	Difficult	Medium
6	**Easy**	**Medium**	**Easy**	Difficult	**Medium**	Medium
7	Medium	Medium	Easy	Difficult	Difficult	Difficult
8	Difficult	Difficult	Medium	V. difficult	Difficult	**Medium**
9	V. difficult	Difficult	Difficult	Medium	**Medium**	Easy

Segment	Batinase ® neutral	Purafect	Protease 6L	Protease 200	Promod	Bromelain
10	V. difficult	Medium	Medium	**Medium**	Difficult	Easy
11	V. difficult	Difficult	Medium	**Easy**	Difficult	Easy
12	Difficult	Medium	**Medium**	Medium	Medium	Easy
13	Medium	Difficult	**Easy**	**Easy**	**Medium**	-
14	**Easy**	Difficult	Medium	Medium	Difficult	-
Control - no enzyme	V. difficult					

("-" denotes no sample)

It is unclear why some wool loosening occurred in association with gel strips that showed no staining in the initial gel. It is possible that some lack of reproducibility occurred between the initial stained gel and the full gel used for the separation of each enzyme. It may be that the estimation of the exact location of the protein in the unstained portion of the gel had shifted relative to the stained marker lane.

Table 11: Depilation results of "Ornstein Davis" pH 4.2 separation of enzymes

Enzyme gel excision	Grain	Epidermis	Upper ORS	Lower ORS	Bulb
Purafect #4	Heavy damage all over surface	None remains	None remains	None remains	None remains
Purafect #6	Medium damage all over surface	Scattered patches remain	Mostly intact	Some breaking up	Some breaking up lots of debris remains
Batinase ® neutral #1	Scattered damage between follicle mouths	Some scattered remnants	Mostly intact	Some breaking up	Mostly intact
Batinase ® neutral #6	Some scattered damage	Some scattered remnants	Mostly intact	Some breaking up	Mostly intact
Batinase ® neutral #14	Heavy damage all over surface	Some scattered remnants	Mostly intact some breaking up	Some breaking up	Mostly intact
Protease 6L #2	Some scattered damage	None remains	Mostly destroyed some debris remains	Mostly destroyed some debris remains	Lots of breaking up some small amount of debris remains

Enzyme gel excision	Grain	Epidermis	Upper ORS	Lower ORS	Bulb
Protease 6L #4	Heavy damage all over surface	Patches remain between follicle mouths	Mostly destroyed	Mostly destroyed	Lots of breaking up some small amount of debris remains
Protease 6L #6	Some scattered damage	Some scattered remnants	Mostly destroyed some debris remains	Mostly destroyed	Lots of breaking up some small amount of debris remains
Protease 6L #12	Scattered damage at follicle mouths	None remains	Mostly intact some breaking up	Some breaking up	Some breaking up
Protease 6L #13	No damage smooth follicle mouths	Intact no sloughing	Intact	Intact	Some breaking up
Protease 200 #4	Heavy damage at follicle mouths	Almost all sloughed off	Mostly intact some breaking up near surface	Mostly intact	Mostly intact
Protease 200 #10	Heavy damage all over surface	Patches remain between follicle mouths	Mostly intact some breaking up	Mostly destroyed some debris remains	None remains
Protease 200 #11	Heavy damage at follicle mouths	Mostly intact some sloughing	Mostly intact	Some breaking up	Some breaking up some debris remains
Protease 200 #13	Heavy damage over all surface	Some scattered remnants	Mostly intact	Mostly intact	Mostly intact
Promod #1	Scattered damage at follicle mouths	Patches remain between follicle mouths	Intact	Mostly intact	Mostly intact
Promod #3	Scattered damage at follicle mouths	Some scattered remnants	Mostly intact some breaking up near surface	Mostly intact	Mostly intact
Promod #6	Medium damage all over	Patches remain between follicle mouths	Mostly intact	Some breaking up	Some breaking up some debris remains
Promod #9	Some scattered damage	Some sloughed off some patches remain	Intact	Some breaking up	Some debris remains
Promod #13	Some scattered damage	Some sloughed off some patches remain	Mostly intact some breaking up near surface	Heavy breaking up	Not visible
Bromelain #1	Mostly intact some damage at follicle mouths	Some scattered remnants	Mostly intact some breaking up near surface	Some breaking up	Some breaking up some debris remains
Bromelain #4	Heavy damage at follicle mouths	Some sloughed off some patches remain	Mostly intact heavy breaking up near surface	Mostly intact	Mostly intact
Bromelain #8	Some scattered damage over all the surface	Some sloughed off some patches remain	Mostly intact heavy breaking up near surface	Mostly intact	Mostly intact

In an attempt to investigate the effects of different components in the enzymes samples in bold (Table 10) were selected for examination using the SACPIC stain method previously described (section 2.2.3.1). Selected samples were chosen at upper and lower extremes of the depilatory active regions. The results of the SACPIC staining are given below in Table 11:

The results showed that fractionation of crude commercially available enzymes did not have the positive effect on the quality of the skin that was hoped for. In other words when a fraction of the enzyme preparation achieved dewooling it was inevitably accompanied by damage to the grain layer. This indicated that a change in the process and/or a change in the enzyme would be required to achieve successful depilated skin and that simply purifying the broad spectrum protease products available would be insufficient.

3.2.3 Enzyme selection – Summary

All of the enzyme formulations tested, except for Bromelain were found to depilate well using the immersion process. This supported the previous findings that general protease activity brings about depilation.

The wool solubility results showed that all the enzyme formulations provided commercially acceptable wool with no significant wool damage. These findings support previous work that wool is resistant to the enzymes used for depilation.

Further purification of the enzymes responsible for the activity in the crude commercial proteolytic enzyme preparations was possible by using electrophoretic techniques, but no fractions were found that could dewool without damage to the grain layer as observed by SACPIC staining. It is likely that the depilation is achieved because of the combination of a number of enzyme actions, some of which damage the collagen structure. Further examination of industrial enzyme preparations were therefore made without further attempts to separate dewooling-active non-damaging components.

The best results were achieved with Purafect 4000 L. Reasonable results were also achieved with Protease 6L

3.3 Characterisation of optimal enzyme depilatory

In order to examine the activity of enzymes against the proteins present in sheep skin an enzyme assay was required.

3.3.1 Assay method

Since skin protein is mostly collagen and since collagen is largely insoluble, determination of enzyme activities against skin protein in general is somewhat difficult. This is because solubilisation of collagen may result in changes in its susceptibility to enzyme attack. Previous authors have described a method for determining the activity of enzymes on a solid substrate based on finely ground powdered hide (Mozersky & Bailey, 1992) (Cantera *et al.*, 1997). Determination of enzyme activity against skin substance was therefore based on this method (see section 2.6). The substrate used in that protocol (Mozersky & Bailey, 1992) was made from bovine material and was generated in a process where hide powder was treated to temperatures in excess of 60°C which would be expected to denature proteins in the skin. So as a result, two substrates were prepared: one using the standard method ((Mozersky & Bailey, 1992)) and the other using ovine material while maintaining temperatures below 60°C.

3.3.1.1 Skin powder azure substrate (SPA)

First, ground degreased skin was prepared as follows: Fresh lambskin was shaved then frozen at –10°C. The frozen skin was shaved again to remove all remaining wool that projected from the surface of the skin. The frozen skin was lyophilised then ground in a Wiley mill #2 mill with 3mm mesh size. The ground material was then degreased in a Soxhlet with dichloromethane for 6 hours at 6 changes of solvent per hour. The skin was then dried at 40°C for 16 hr in an oven.

The colorimetric substrate was prepared from the ground degreased skin using the following method; 60g of ground degreased skin was fully wetted in a solution of 400 mL of 0.45% sodium chloride under vacuum. The solution pH was then adjusted to pH 11 using 0.5 M tri-sodium orthophosphate and 6g of Remazol Brilliant Blue ‘R’ (Sigma) was added. The mixture was then stirred for 30 minutes at 40°C. Then either 800 mL of water at 60°C was added and the mixture stirred for another hour at 45°C (designated low temperature, SPA < 70) or 800 mL boiling water was added and the mixture stirred at 75°C for 5 minutes (designated high temperature, SPA>70).

The mixtures were cooled and settled over 1 hour. The supernatant water was siphoned off and another 800 mL of cold water was added and mixed. These washes continued until the supernatant water appeared clear and showed no peak of absorbance at 595nm.

The resultant "blue" slurry was rinsed with 20 successive volumes of acetone. The dewatered solids were then dried at 40°C overnight. The dry solids were ground in a coffee grinder in the presence of solid carbon dioxide and sieved. The solids fraction between 90-200 mesh sizes were kept. This "skin powder azure" (SPA) was then stored at room temperature.

3.3.1.2 Skin powder azure assay

100 mg of SPA was measured into a conical flask. 15 mL of sodium carbonate/sodium bicarbonate buffer added and shaken on a shaking water bath in a 50 mL conical flask at 2 Hz at 35°C unless specified otherwise. The mixture was allowed to equilibrate for 5 minute then a 5 mL of enzyme solution was added resulting in a final buffer concentration of 0.5 M sodium carbonate/sodium bicarbonate at pH 10.5 unless otherwise specified.

The enzyme solution was prepared immediately before use; 1g of protease (Purafect 4000L, Genencor Int) was made up to 25 mL with double distilled water. A 5 mL aliquot of enzyme solution was added to the SPA slurry to begin the assay resulting in final concentrations of: 5 g/L SPA, 10 g/L stock enzyme, in 20mL of 0.5 M carbonate buffer at pH 10.5. 1 mL samples were removed from the slurry at 10 second intervals for 1 minute then a further sample at 2 minutes. The enzyme reaction in each sample was quenched by addition to 2 mL of 20% trichloroacetic acid. The samples were then centrifuged at 2000g for 30 min and the absorbance (AU) at 595 nm of the supernatant measured on a Cary 500 UV/vis spectrophotometer.

3.3.1.3 Skin powder azure assay results

Duplicate tests were carried out on high temperature SPA (SPA >70), low temperature SPA (SPA<70) and Sigma Remazol brilliant blue R - dyed hide powder (HPA – Sigma, H6268-1G) to compare different substrates. Each test was carried out at 35°C using 0.5

M sodium carbonate/bicarbonate buffer at pH 10.5. The results are illustrated below in Figure 23

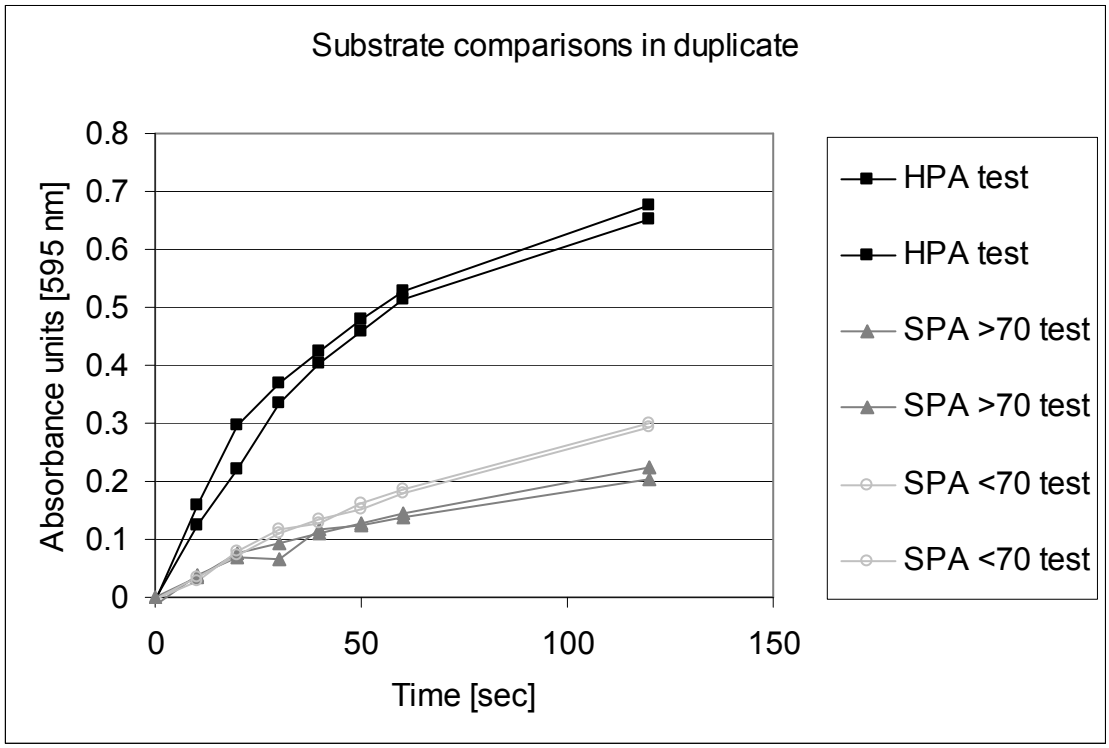


Figure 23: General skin substance assay. A comparison of duplicate tests using Purafect for each of the experimental substrates was examined

Control samples taken both before and after the 5 minute equilibration period in the absence of any enzyme showed no significant dye release (absorbance <0.02). This is important for the "low temperature" SPA since it indicated that the dye was properly bound and was not released in the absence of enzyme.

In the presence of a proteolytic enzyme the low temperature SPA powder gave a smooth response of absorbance vs. time. Although the dye release was not as high as for dyed hide powder the response was sufficient to be able to characterise the enzyme activity. The use of ovine skin powder was preferred over the bovine material to ensure the closest relevance to lamb skin processing.

It can also be seen that the rate of dye release with time was not linear. Attempts were made to fit simple zero, first, and second order equations to the data but the fits were not good (results not shown). This was aimed at an understandingof the full enzyme kinetic system and may be a fruitful avenue of future research. For the purpose of this

examination of enzyme activities against process parameters such as temperature and pH the initial rate was of most interest. In order to determine the initial rate two different non linear equation fits were applied:.

$$y = \frac{a \times t}{b + t}$$

Equation 1: two term regression equation

$$y = \frac{a \times t}{b + t} + c$$

Equation 2: three term regression equation

These two methods provide a regression output from which the initial slope could be calculated. The regression fit for a typical SPA assay is illustrated in Figure 24.

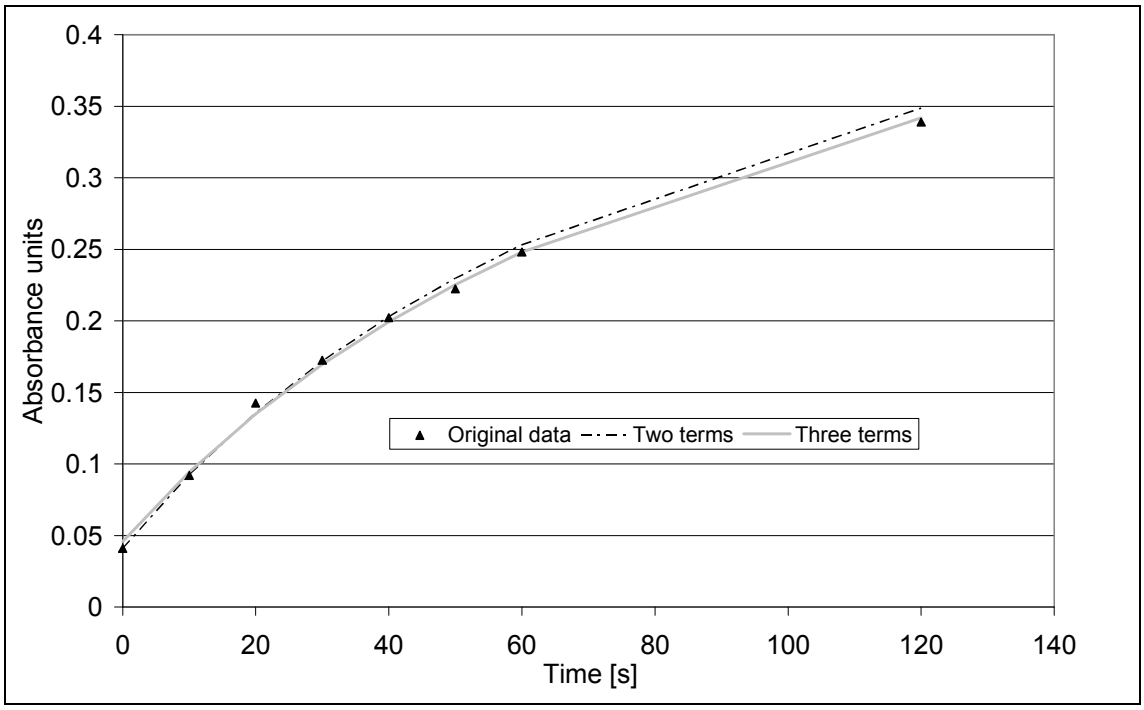


Figure 24: Skin powder azure assay regression The original raw absorbance data is shown as black triangles. The predicted plot from the equation with two terms is show as a dashed black line The prediction using the three term regression equation is shown in grey.

Figure 24 shows that the equation with three terms gave a qualitatively better description of the data than the two term equation. To determine the slope of the three

term non linear fitted line at time zero the equation of the fitted line (**Equation 2**) was differentiated to give Equation 3.

$$y' = \frac{a}{b+t} - \frac{a \times t}{(b+t)^2}$$

Equation 3: Differentiated form of the Equation 2

Equation 2 can then be solved at time = 0 giving.

$$y'_{(0)} = \frac{a}{b}$$

Equation 4: Initial slope of the SPA dye release fitting equation

When the SPA data was fitted using equation 2 the initial reaction rate could be calculated from a/b (equation 4) where a and b represent the coefficients in the fitted equation. The results of fitting the data and calculating the initial slope are illustrated in Figure 25.

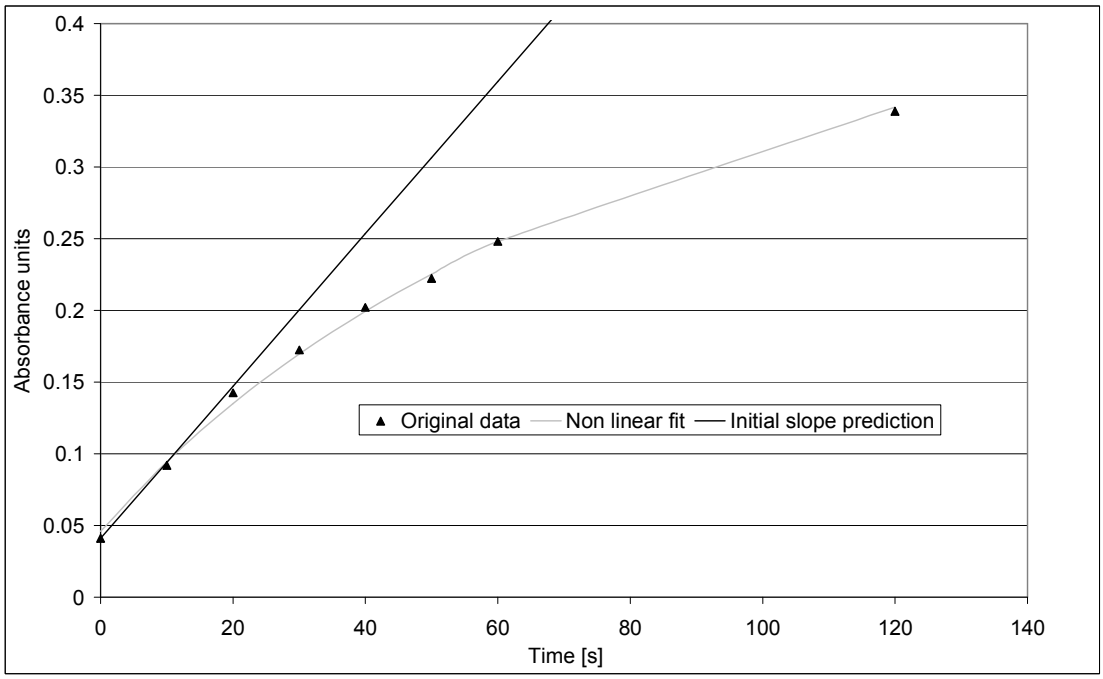


Figure 25: Skin powder azure assay for initial slope estimation of the SPA assay using a non linier fit. The original raw absorbance data is shown as black triangles. The prediction using the non-linear regression is shown in grey. The predicted initial slope is then illustrated by the black line.

It can be seen in Figure 25 that the predicted initial slope generated using non-linear least squares regression does estimate the initial slope of the original data as observed

by eye. The initial rate was therefore determined by fitting the three term equation and then using equation 4 for the remainder of the work using SPA.

3.3.1.4 Assay error

The error in the enzyme assay using SPA was determined by conducting 6 replicates at 35°C, pH 10.5. Each test had 8 data points for a total of 48 data points used to estimate the error of the slope. The data was fitted to the non-linear equation (**Equation 2**). The standard error of each of the parameters was estimated using the "jack-knife" procedure (Harris, 1998). The results from this procedure are given in Table 12.

Table 12: SPA error estimation

	a	b	c
Parameter values	0.50	107	0.602
Standard error	0.04	15	0.004

The initial slope from this replicate experiment can then be calculated from

Slope = a/b = 0.50/107 = 0.0046 AU/s

The standard error in this example is then the geometric sum of the relative errors of each parameter. i.e. $((0.04/0.5)^2+(15/107)^2)^{0.5}$ = 16 %

The SPA test provides results with a standard error of about ± 0.0007 [AU/s] at 8 data points per test. This results in a measurement error of 0.0018 AU/s

3.3.2 Effect of pH

3.3.2.1 pH effect on measured activity

A number of different pH buffers were prepared and used in the SPA assay described above. Each buffer was prepared at 0.5 M and the final pH was measured using an Orion pH meter using standards at the following std buffer pHs 1.68,4.00,6.88,9.22 with a fit of 98.5%. The buffers used are given below in Table 7. Since the buffer capacity of a buffer depends on the salts used and the target pH a range of buffer salts were used to ensure that a range of ultimate assay pHs were tested.

Table 13: Buffers for pH influence on skin powder azure assay

Buffer	final pH
Boric acid/ Sodium Hydroxide	7.5
Glycine/ Sodium Hydroxide	8.6
Boric acid/ Sodium Hydroxide	8.88
Glycine/ Sodium Hydroxide	9.75
Sodium Carbonate/sodium bicarbonate	9.9
Glycine/ Sodium Hydroxide	10.2
Sodium Carbonate/sodium bicarbonate	10.36
Glycine/ Sodium Hydroxide	10.36
Glycine/ Sodium Hydroxide	12

The effect of pH on the initial rate of the SPA assay are illustrated in Figure 26.

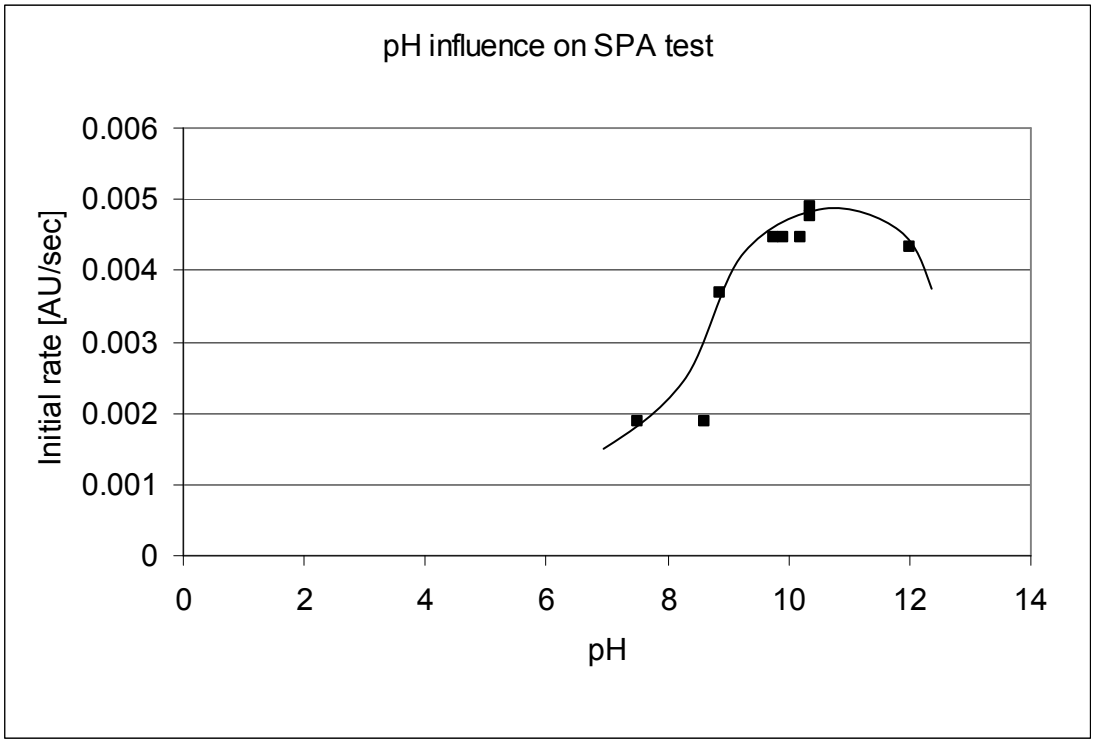


Figure 26: Effect of pH on the skin powder azure assay (Purafect)

The results showed that the optimal activity of Purafect was between pH 10 and pH 11. Since the assay represents an activity against the materials found in ovine skin, it follows that an enzymes optimal pH in this assay is likely to be associated with its optimal pH during depilation. To confirm the optimal pH for depilation using this enzyme a further experiment was carried out (see section 3.3.2.2).

3.3.2.2 pH effect on depilatory effectiveness

It has been reported that enzyme depilation should be carried out at higher pHs (Brady *et al.*, 1989b).The tests undertaken to determine the pH dependence of the enzyme showed it had optimal activity between pH 10 and 11 Therefore experiments to determine the effect of pH on enzyme depilation were carried out at pH 9.5, 10.5, and 11.5.

The same method and enzyme was used as described in section 3.2.1. A buffer was adjusted to the following pHs: 9.5, 10.5, and 11.5 using a 0.5 M sodium carbonate/sodium bicarbonate system. Although the carbonate buffer system is at the limit of its range at pH 11.5 the pH of each skin piece was measured after the experiment and found to be at the pH desired. The depilation efficiency was measured as described in section 3.2.1.2 at each of these pH values and the results are shown in Figure 27.

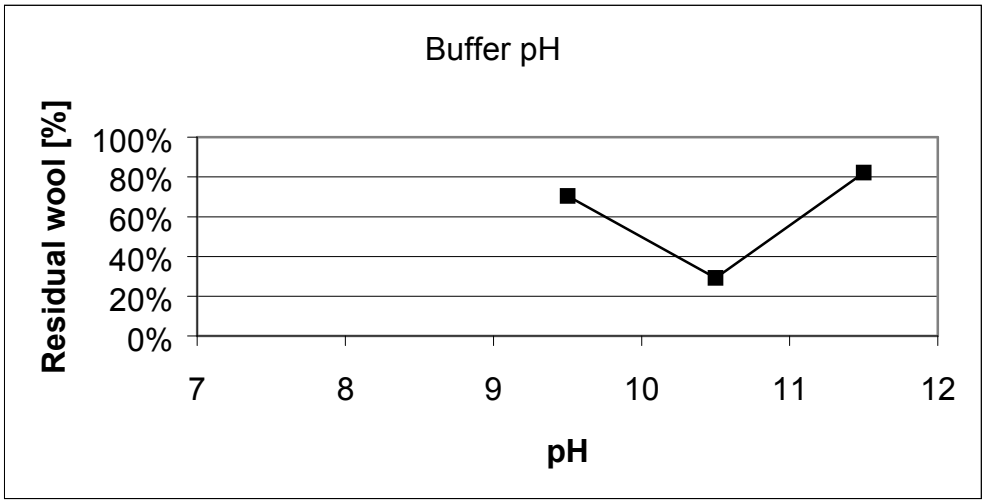


Figure 27: Depilation pH response

It can be seen that pH 10.5 gives the best level of wool removal. This agrees well with the results found using the SPA assay where the overall activity of the enzyme had a maximum at this pH when examined with a variety of buffer salts as shown in Figure 26.

3.3.2.3 Quenching enzyme activity

In addition to determining the optimum operational pH it was also important to determine the pH at which the enzyme depilatory had no activity against skin substance.

In order to stop the enzyme reaction and halt depilation the skin could be immediately pickled as per the standard process (Appendix 8.2.3). The pH at which the alkaline depilatory enzyme began to lose its activity was required along with the pH at which the enzyme was rendered effectively inactive. These parameters were determined in a series of experiments.

0.05 M/0.05 M citrate/phosphate buffers were prepared with the following pHs ranging from 2 – 7. Then 0.4 mL of Purafect was dissolved in 10mL of each buffer and incubated at 25°C for 0 or 1hr before the SPA assay was carried out. After this time pH was again checked to ensure that any buffer salts existent in the enzyme reagent did not effect the desired pH of the assay. For the assay a 5 ML sample of treated enzyme was added to 15 mL water and the residual activity determined using the SPA assay developed in sections 3.3.1 with the exception that the buffer as altered as described above. The results for each assay set are illustrated in Figure 28.

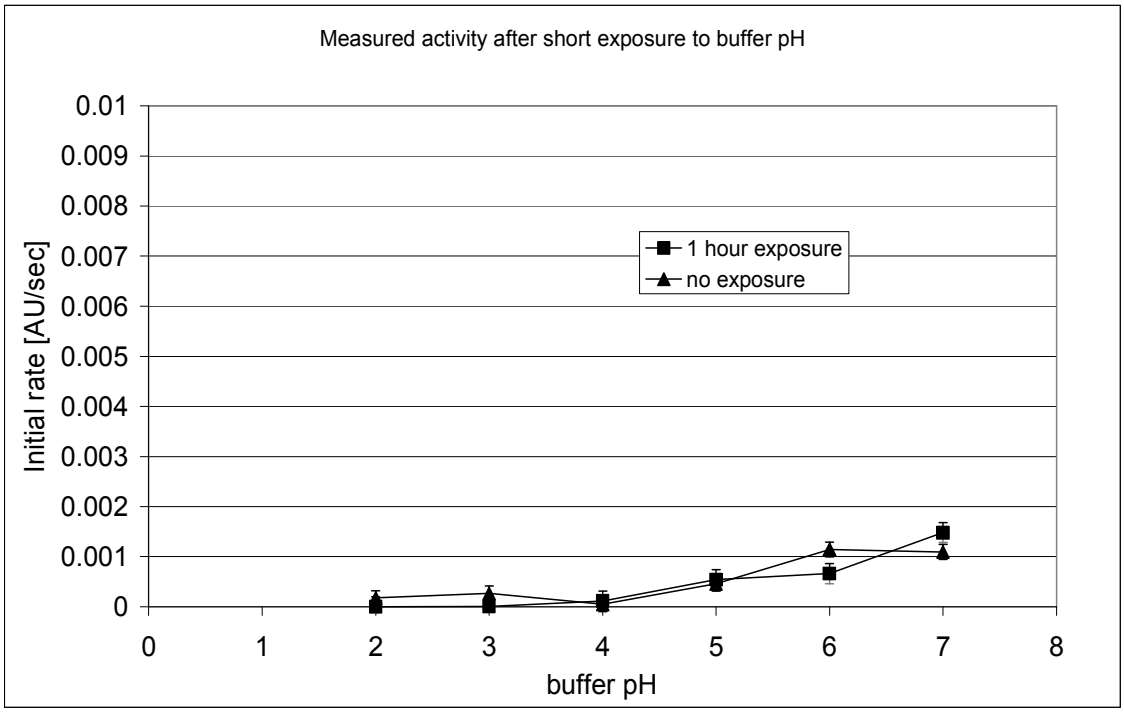


Figure 28: Influence of low pH on enzyme activity against general skin substance as measured by the skin powder azure test

The results show that the initial level of activity against general skin substance was negligible at a pH of 4 or below even if left to incubate at that pH for 1 hour. This indicates that enzyme activity can be halted in skin if the pH is dropped below 4.

However it does not indicate whether the activity can be regenerated if the skin pH is subsequently neutralised.

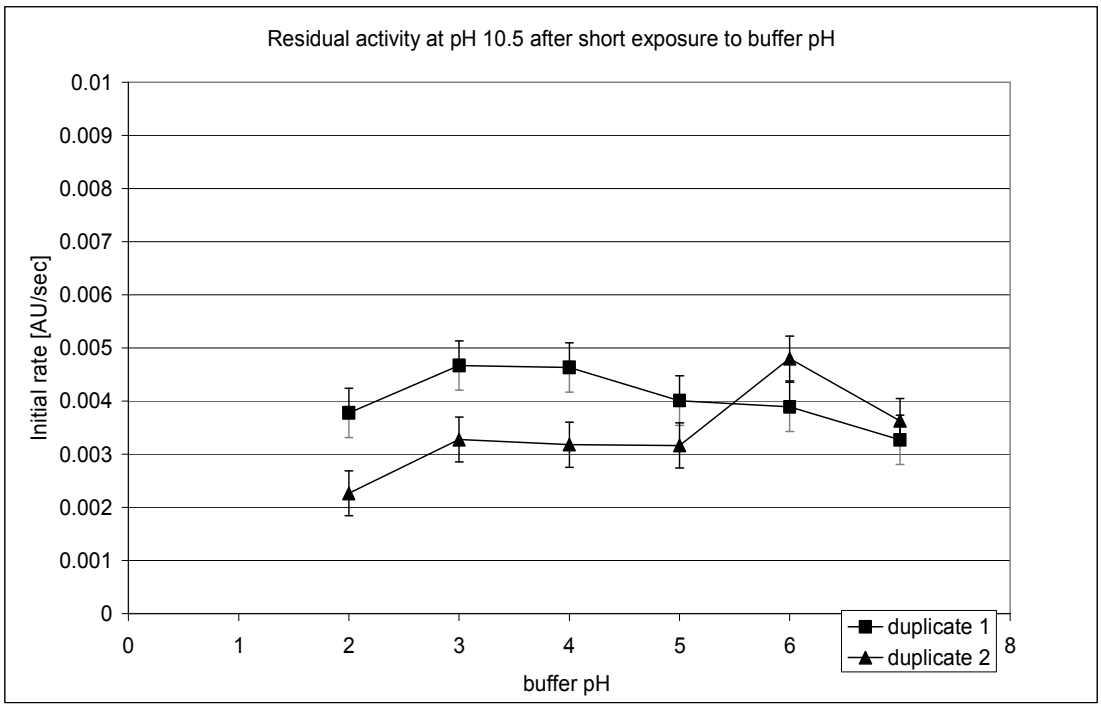


Figure 29: Regeneration of activity as measured at pH 10.5 after 5 seconds exposure to low pH, x axis represents the exposure pH, y axis represents the activity of the enzyme at pH 10.5

To determine the pH and time of exposure required to permanently destroy the activity of the enzyme further experiments were carried out. Here the enzyme was first exposed to a given pH for a period of time (5 seconds, 2 hours, and overnight) at 35°C and then the enzyme assay was carried out at the optimal pH of the enzyme (pH 10.5). To achieve this a 1mL sample of enzyme was made up to 25 ML in 0.05 M Citrate/Phosphate buffer at the test exposure pH and then held for the exposure period at 35°C. 15 ML of buffered SPA slurry was prepared as described in section 3.3.1 and equilibrated at 35°C for 5 minutes. A 5 ML sample of the pretreated enzyme was then added to the SPA slurry resulting in a final pH of 10.5 in a 0.5 M sodium carbonate/ sodium bicarbonate buffer. After conclusion of the assay the pH of the remaining slurry was assessed and found to be at 10.5 as desired.

The enzyme activity as assessed at pH 10.5 after a period of exposure to a range of pHs is shown in Figure 29, Figure 30, and Figure 31 for 5 seconds, 2 hours, and 24 hours exposure respectively.

These results show that a short exposure to low pH had little effect on the activity of the enzyme. While the enzyme, when assayed at a given pH, had negligible activity, the enzyme that was exposed to pH 4 for a short period (5 seconds) was essentially unaffected when tested at the optimal pH of 10.5.

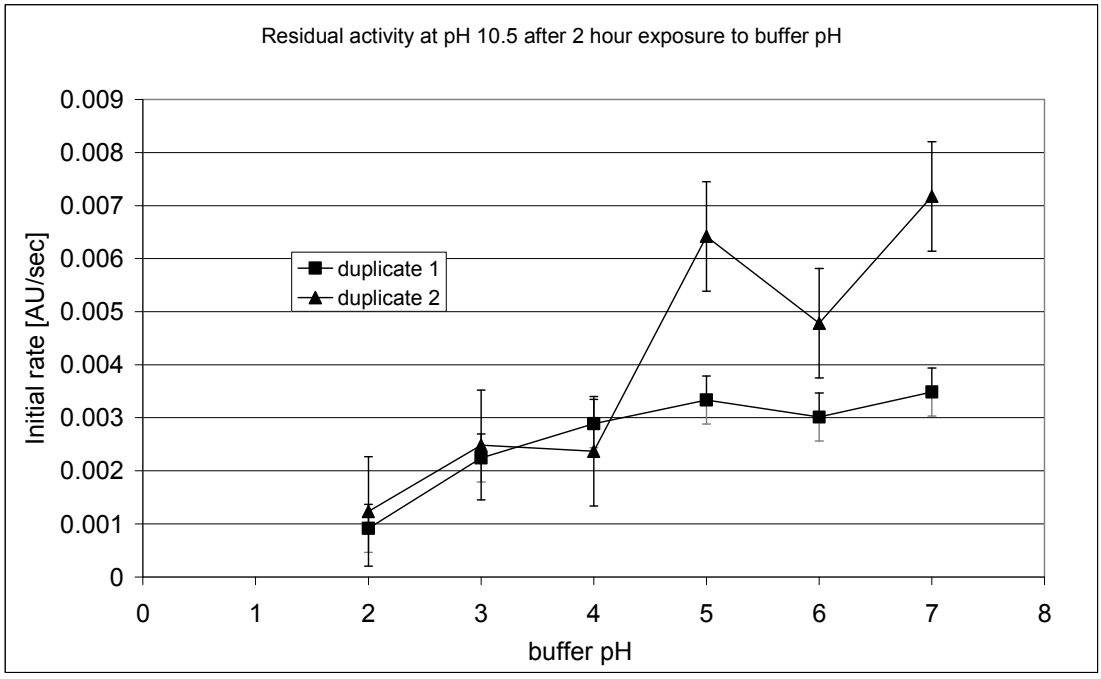


Figure 30: Regeneration of activity in pH 10.5 after 2 hours exposure to low buffer pH, x axis represents the exposure pH, y axis represents the activity of the enzyme at pH 10.5

The results show that while measured activity under pH 4 conditions was effectively quenched, considerable exposure time at that pH was required to ensure an effective reduction in enzyme activity. Exposure for 2 hours at that pH was not enough to effectively eliminate enzyme activity. However, when the enzyme was exposed to pH 3 or below for a period of 24 hours, such as would occur during a standard pickling process, then the residual activity against general skin substance was negligible.

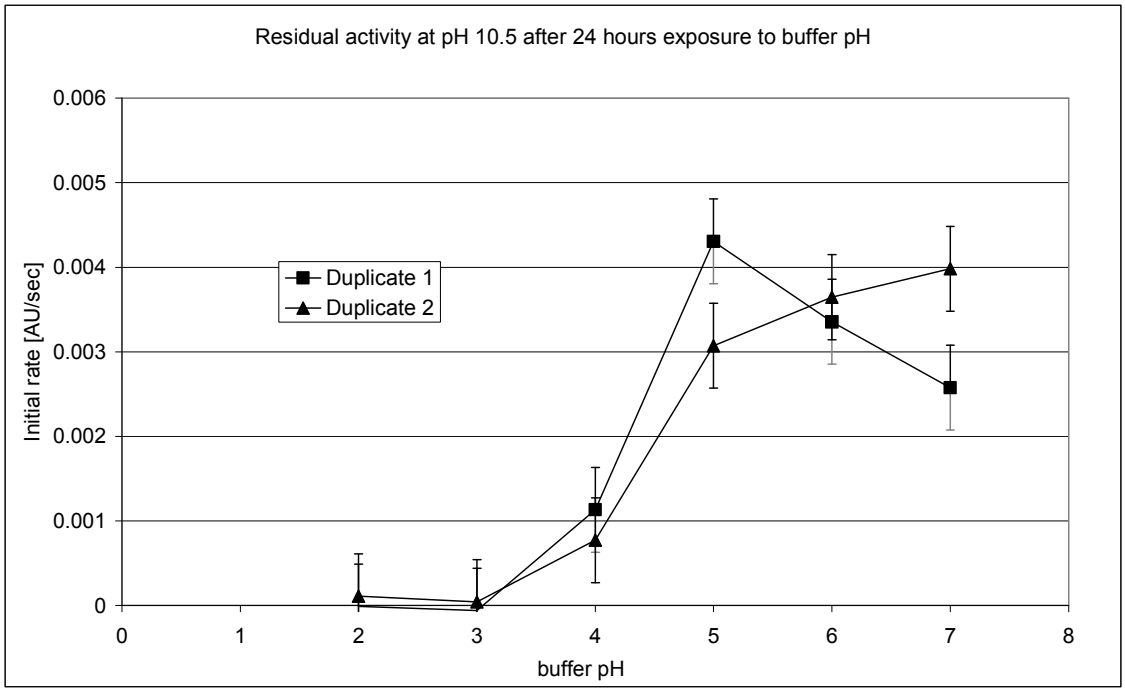


Figure 31: Regeneration of activity after 24 hours exposure to low pH, x axis represents the exposure pH, y axis represents the activity of the enzyme at pH 10.5

These results indicate that damage to the skin substance would not be caused by continuing activity of the depilatory enzyme during or after the standard pickling process.

3.3.3 Effect of temperature

Like all chemical reactions enzyme activity is dependent on temperature (Levenspiel, 1999). The optimum temperature for Purafect 4000L is quoted by manufacturers to be 60°C. A preliminary examination of temperature effects on enzyme depilation was carried out at 35°C, 20°C, and 10°C. The methods for enzyme application and solution formulation were as described in section 3.2.1.

At 35°C excessive damage to the skin was evident and furthermore, depilation was incomplete. At 10°C depilation was still incomplete after 16 hours of treatment.

In order to assess activity changes due to temperatures, the enzyme assay was carried out over a range of temperatures using 0.5 M sodium carbonate/bicarbonate buffer (pH 10.5). The results are illustrated in Figure 32.

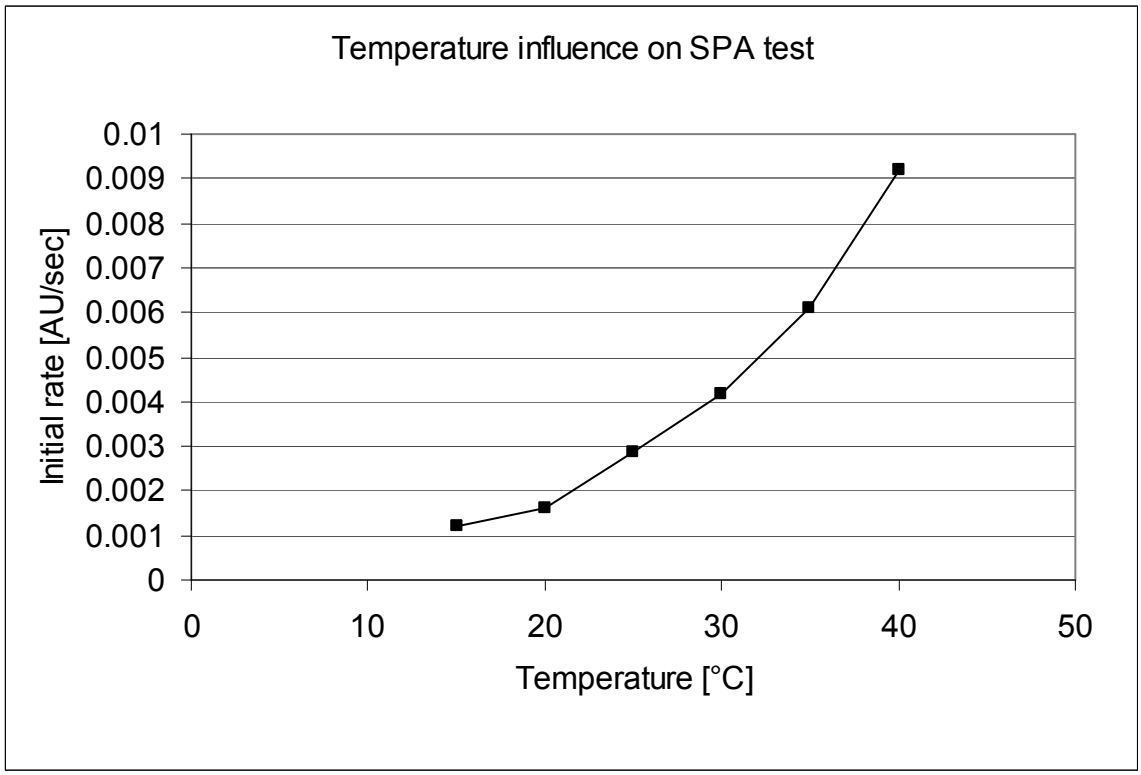


Figure 32: Effect of temperature on enzyme activity

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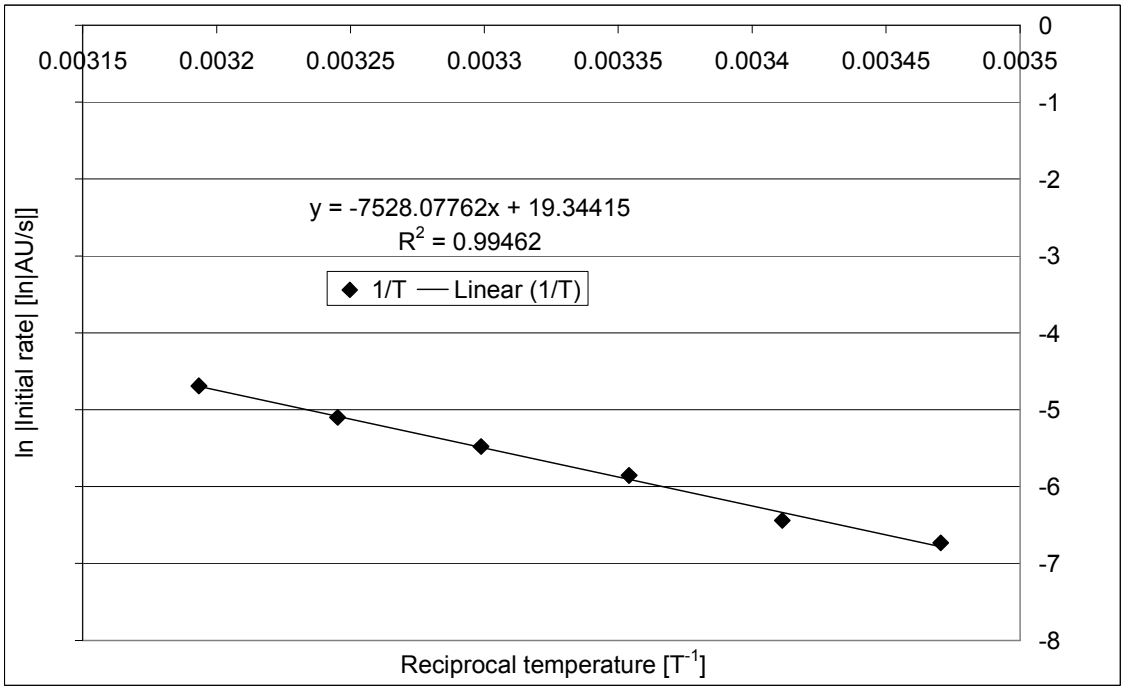


Figure 33: Transformed rate vs. temperature plot indicating the activation energy of the enzyme. Assuming an Arrhenius relationship the slope is –E/R where: R is the gas constant (8.314 J.mol^{-1}.K^{-1}), E is the activation energy (63000 J.mol^{-1}) (Levenspiel, 1999)

Controls containing no enzyme were again measured at each of the temperatures and were found to have less than 0.0005 AU/s. Results showed that there is an increasing activity with increasing temperature over the range investigated. By plotting the ln(rate) vs. 1/T where T is in degrees Kelvin an estimation of the activation energy of the initial rate can be made. This transformation is illustrated in Figure 33

The activation energy of the initial reaction rate was calculated to be 63000 $J.mol^{-1}$. This means that a shift of about 8°C from 20°C would result in a change in reaction rate of a factor of 2. It is important therefore that subsequent dewooling experiments be carried out at a constant temperature to ensure consistency.

High levels of damage were noted in preliminary experiments carried out at 35°C and since at 35°C the enzyme had three times the level of activity in comparison to 20°C (which was the base temperature for depilation used throughout this work) these results suggest that altering the temperature of the depilation system is likely to result in either severe damage or incomplete depilation. Simply halting the process once depilation is complete is unlikely to be successful in stopping all the damage if careful controls on the temperature are not maintained. It was determined therefore that both an investigation on the variability in enzyme depilation in practice and the cause of damage be carried out.

3.3.4 System optimisation - Summary

General enzyme activity against the proteins in sheep skin can be measured with a simple chromogenic assay using the ovine skin based substrate developed in this work (section 3.3.1).

pH had a significant influence on the activity of the enzymeagainst ovine skin substrate as would be expected and the use of pH to eliminate the enzyme activity at the end of the depilation process could be effective.

Temperature also affected the activity against the general skin substrate and may be a useful means to modulate the activity of an enzyme depilation process. Careful control of the temperature is required to ensure repeatable experimental results.

3.4 Enzyme characterisation - Conclusions

This chapter presented work on characterising enzymatic depilation and the nature of the faults found in the product after enzymatic depilation. From this an investigation into the process of enzymatic depilation damage was carried out to characterise the factors that were responsible for poor depilation and pelt damage due to enzyme depilatory reagents. These aims were successfully achieved with the following findings:

- Progressive damage to the collagen occurred with a range of enzymes investigated. Some of the damage caused to the skin surface may be reduced by either reducing pelt variability or improving process specificity. Some damage to the grain surface seems inevitable using a selection of broad spectrum proteases as previously suggested (Brady *et al.*, 1990).

- Achievement of successful depilation will depend on the availability of an enzyme that can specifically target the hair root sheath cells and the "cementing" substance that glues the hair in place. As enzyme activity is affected by temperature and pH successful enzymatic depilation will require well controlled temperature and pH for both achieving the wool loosening process and stopping subsequent undesirable enzyme activity.

- Determining the barriers to enzyme penetration and depilation action will assist in determining the causes of pelt variability, the targeting of which will assist the depilation process by reducing the risk of overexposure of the desirable pelt proteins to non-specific activities of the depilatory enzyme.

Variation in enzymatic depilation

4.1 Introduction

While exact details of the short comings of enzyme depilation are scarce some previous authors have reported inconsistent or heterogeneous depilation results on lambskins (Hawkins & Wassenberg, 1997); (Green, 1952). It is likely that some of this could be explained by inherent variation in the skin. Variation in the physical properties used to define the leather textile has lead to the use of an official sampling position on the pelt, so that processes and skins can be compared with the least interference from skin variation (James & James, 2002).

Previous work has shown for calf skin, that the collagen at the surface is still intact when the cementing components required to loosen hair have been removed (Stirtz, 1965). However, surface damage continued to occur after this time as a result of subsequent processing. In order to ensure complete depilation over the whole skin, enzyme exposure can be extended but this inevitably results in damage to those parts of the skin which are “over exposed”. Alternatively the enzyme exposure can be reduced which, while protecting the skin from enzyme damage, risks incomplete depilation.

Knowledge of the heterogeneity of the skin structure and composition could explain some of the heterogeneity of depilation and surface damage, offering some insight into how to achieve a more uniform process.The variability of depilation during the conventional lime/sulphide process is well known and dealt with through correct application of the lime/sulphide depilatory to the flesh side of the skin. However, the variability of enzyme depilation has not been previously described and there was no reason to accept thatthe same pattern of depilation would occur as in the conventional process nor to blindly apply the same solution.

The aims of this chapter were therefore to characterise the variation in enzymatic depilation and damage across the skin and to compare this to the heterogeneity in skin composition found within a single lambskin. By determining which parameters correlated with depilation, mitigating conditions may be found to provide efficient depilation across the whole skin while at the same time minimizing the risk of

proteolytic damage to more susceptible areas of the skin. Possible causes for heterogeneous depilation investigated in this work were: skin thickness, grease content, wool fibre diameter, and the strength with which wool fibres were held in their root sheaths.

4.2 Heterogeneity of depilation

In section **3.2** different proteolytic enzymes were examined for their ability to depilate lambskin. It was found that all of the enzymes investigated resulted in depilation and damage of the skin surface of lambskin to some extent. In order to characterise the depilation process and its associated problems, the best performing enzyme (Purafect 4000L) was selected to characterise variations in depilation time and damage across individual lamb skins.

4.2.1 Depilation method

Duplicate lambskins were prepared for enzyme (Purafect 4000L) depilation according to the method summarised in section 3.2.1. Each skin was exposed to 4 volumes of enzyme solution in a drum rotating at 4 rpm, then held in contact with the enzyme solution at 20°C for 16 hours with the ease of depilation tested every hour. Photographs were taken each hour to illustrate the extent of depilation observed and to show the locations where the depilation occurred.

4.2.1.1 Depilation progression results

Figure 35 shows that enzyme depilation proceeds quickly in the flank regions of the skin and only relatively slowly in the backbone region. During the assessment of depilation there was a clear distinction between the region of skin in which the wool had become loosened and the region in which it had not. There was no band between loosened and tight wool where partially loosened wool was observed. The overall variability observed here is similar to that observed during conventional lime sulfide depilation which is attributed to structural differences in the skin in those regions (Vivian, 1978). This raises the questions as to when grain surface damage begins to occur, and whether or not it is related to this observed variability in depilation.

By photographing skins as they became depilated then manually counting the pixels of the depilated region and comparing to the number of pixels of the whole skin the proportion of area that has been depilated can be plotted against time. This data is illustrated in Figure 34.

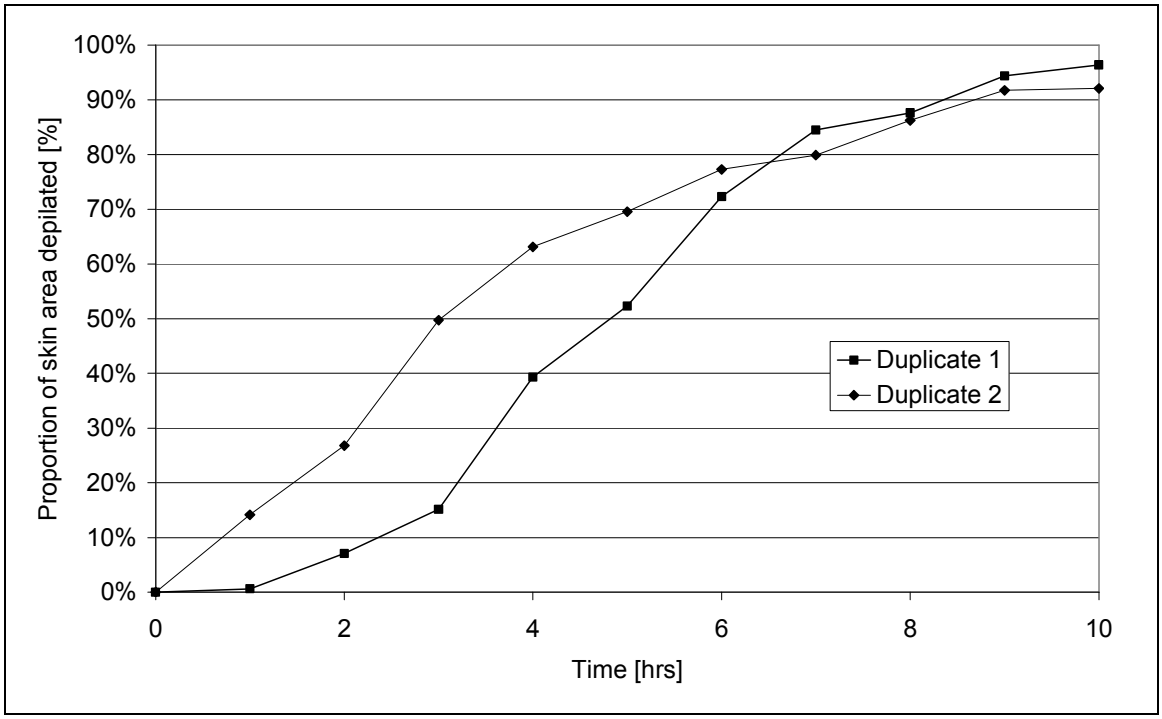


Figure 34: Progression of enzymatic depilation. The first regions to depilate are the flanks. As depilation proceeds the regions of depilated area grow in size until the back bone is all that is left. The backbone and neck depilate last.

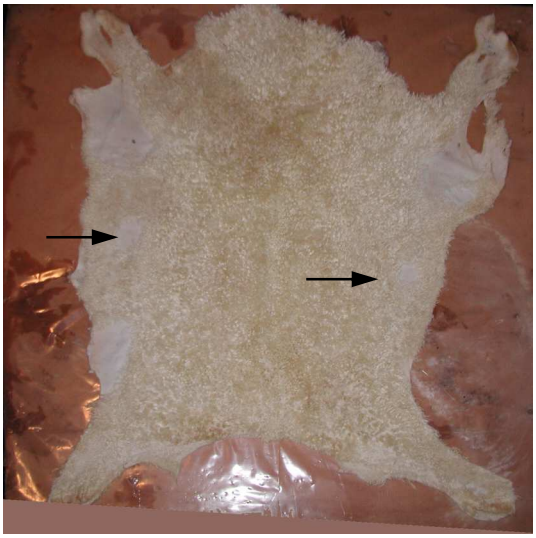
The progression of the enzymatic depilation follows a sigmoid curve in general. Regions which are easily loosened depilate very early on. Then the bulk of the wool is progressively loosened until the end of depilation where some small regions take particularly long to depilate.

The shape of this curve has significant implications to the quality of the final product. If damage occurs progressively after depilation is complete then regions of the skin which are depilated in the first hour will be exposed to the enzyme for a further 9 or more hours while the remainder of the wool on the skin becomes loosened. However, if by manipulation of the skin or the conditions of the depilation the shape of the curve could be altered so that it was steeper and the rate of depilation across the skin more even,

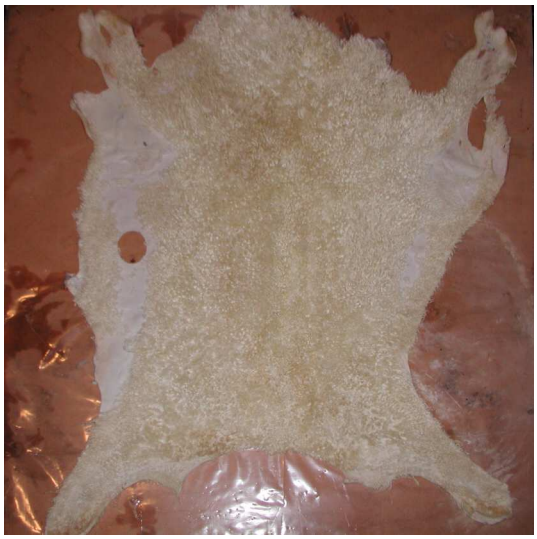
then the enzyme reaction could be halted sooner after the initial depilation thereby reducing the risk of subsequent damage. It would be useful at this point to know the time scale within which the depilation needed to be complete. This then would define the width of the sigmoid depilation curve required to achieve a successful depilation with minimal damage.

0hr

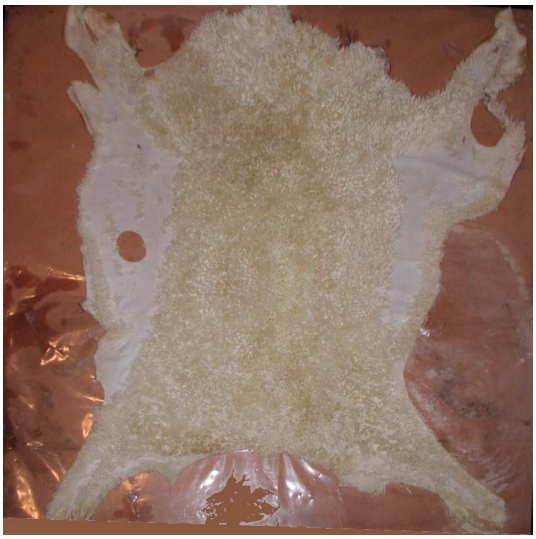
1hr



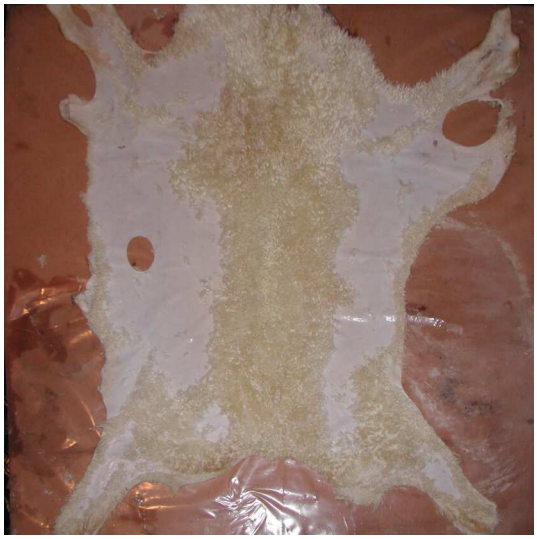
2hr



3hr



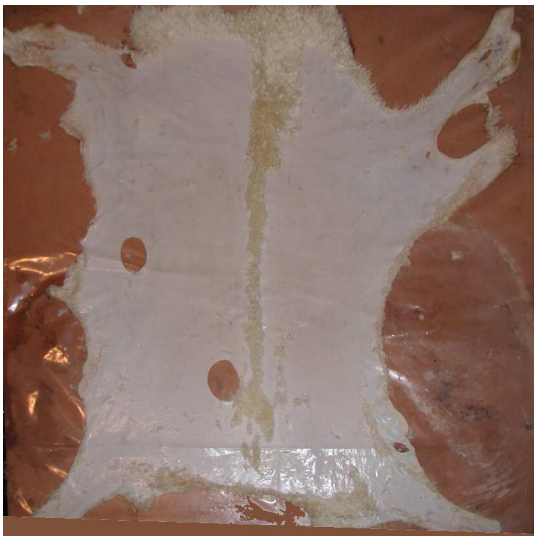
4hr



5hr

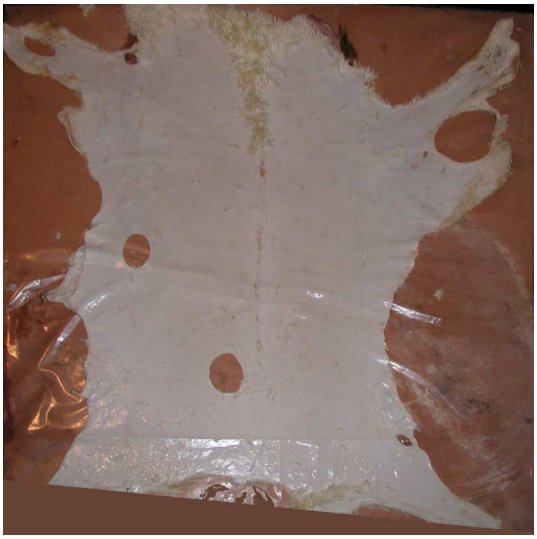
6hr

7hr



8hr

9hr



10hr

Figure 35: Enzyme depilated skin: beginning at 0hrs with 1hr intervals. The results from the duplicate skin match the results observed for the illustrated skin (the holes in the skin are sample holes) Note depilation in flanks first (arrowed)

Examination of the grain surface showed that less damage had occurred to the backbone region (late depilation region) in comparison to the flanks (early depilation region), indicating there was, as expected, a correlation between surface damage and ease of depilation. The question remained whether the proteolysis required for dewooling precedes damage of the grain layer at any location on the skin as found for calf skins by (Stirtz, 1965). If so, then the possibility exists for dewooling without damage if the rate of dewooling could be made more uniform across the skin area. Alternatively enzyme activity could be occurring both at the wool fibre sheath and in the grain layer simultaneously, in which case some degree of damage will be expected no matter how uniform the dewooling process can be made unless greater control or selectivity of the enzyme is achieved. Such control could be gained by using a more targeted enzyme or by controlling the conditions of the depilation process to reduce damage.

4.2.2 Grain surface damage

To explore this, an experiment was carried out to monitor the damage to the grain surface in regions of skin that had been depilated for different times. Two lambskins were obtained fresh from the cooling bath at a cooperating meat processing plant. From these skins the flank region and back bone region were excised. The pieces were then treated to the same depilation procedure as described in section 3.2.1

The pieces from the backbone region were held for 16 hours then the wool was pulled. Two depilation regimes were used: the pieces from the flank region were divided into two parts. Depilation after 5 hours (designated as “immediate”) and depilation after 16 hours (designated as “delayed”). Immediately after depilation all the pieces were pickled in the LASRA standard pickle with the addition of 2% formic acid. The skins were then processed through to crust leather using the standard LASRA process (See Appendix sections 8.2.5, 8.2.4, and 8.2.6). The crust samples were then observed under light microscope to characterise the level of damage that occurred.

The experiment was carried out three times and the results from examination of the grain surface of each piece are described in Tables 14 to 16.

Table 14: Grain quality after enzyme wool depilation (Experimental replicate 1)

Skin/Position	Procedure	Interfollicular pads	Follicle mouths
1/Left flank	Immediately depilated	Inconsistent damage (some regions damaged some undamaged)	Inconsistent damage (some regions damaged some undamaged)
1/Left flank	Delayed depilation	Heavily damaged	Heavily damaged
1/Backbone-neck	Delayed depilation	Some undamaged regions surrounding non-depilated regions	Some undamaged regions surrounding non-depilated regions
1/Backbone-butt	Delayed depilation	Some undamaged regions surrounding non-depilated regions	Some undamaged regions surrounding non-depilated regions
1/Right flank	Immediately depilated	Inconsistent damage (some regions damaged some undamaged)	Irregular follicle mouths
1/Right flank	Delayed depilation	Heavily damaged	Heavily damaged
2/Left flank	Immediately depilated	Inconsistent damage (some regions damaged some undamaged)	Inconsistent damage (some regions damaged some undamaged)
2/Left flank	Delayed depilation	Inconsistent damage (some regions damaged some undamaged)	Inconsistent damage (some regions damaged some undamaged)
2/Backbone-neck	Delayed depilation	Inconsistent damage (some regions damaged some undamaged)	Irregular follicle mouths
2/Backbone-butt	Delayed depilation	Inconsistent damage (some regions damaged some undamaged)	Irregular follicle mouths
2/Right flank	Immediately depilated	Some scuffing	Regular / some scuffing
2/Right flank	Delayed depilation	Some scuffing	Slightly irregular/ some scuffing

The results of the first two replicates were not consistent. In general skin samples pickled soon after wool release (i.e. immediately depilated flank samples or samples from the backbone-butt area) had less damage than those flank samples left 11 hours longer than necessary to remove the wool. These results were not always observed however. In some samples heavy damage was observed irrespective of depilation time. In other flank samples only modest levels of damage was observed irrespective of depilation time. For this reason the experiment was repeated giving the results shown in Table 16.

Table 15: Grain quality after enzymatic depilation (Experimental replicate 2)

Skin/Position	Procedure	Interfollicular pads	Follicle mouths
1/Left flank	Immediately depilated	Heavy damage	Heavy damage
1/Left flank	Delayed depilation	Mostly undamaged	Irregular, Some damage
1/Backbone-neck	Delayed depilation	Scattered damage, some undamaged	Some damage, some irregular
1/Backbone-butt	Delayed depilation	Scattered damage, some undamaged	Some damage, some irregular
1/Right flank	Delayed depilation	Very heavy damage	Barely discernable
1/Right flank	Immediately depilated	Very heavy damage	Barely discernable
2/Left flank	Delayed depilation	Slight flattening, scattered heavy damage	Some damage, some irregular
2/Left flank	Immediately depilated	Damaged	Heavy damage
2/Backbone-neck	Delayed depilation	Mostly undamaged	Some damage
2/Backbone-butt	Delayed depilation	Mostly undamaged	Irregular, Some damage
2/Right flank	Immediately depilated	Mostly heavy damage	Heavy damage
2/Right flank	Delayed depilation	Very heavy damage	Barely discernable

Table 16: Grain quality after enzyme wool loosening (Experimental replicate 3)

Skin/Position	Procedure	Interfollicular pads	Follicle mouths
1/Left flank	Delayed depilation	Most pads remain	Some damage to follicle mouths
1/Left flank	Depilated by sulfide after enzyme exposure	No residual wool severe damage, much of the surface removed	Severe damage
1/Backbone-neck	Delayed depilation	Heavy damage all over some very small regions of intact grain enamel immediately adjacent to residual wool	Completely eroded
1/Backbone-butt	Delayed depilation	Medium damage all over some very small regions of intact grain enamel immediately adjacent to residual wool	Completely eroded
1/Right flank	Depilated by sulfide after enzyme exposure	No residual wool severe damage, much of the surface removed	Severe damage
1/Right flank	Immediately depilated	Some damage in follicle mouths much of interfollicular pads still viable some regions of heavy damage much epidermis remains	Some damage

Skin/Position	Procedure	Interfollicular pads	Follicle mouths
2/Left flank	Immediately depilated	No depilation observed at all on this piece	No depilation observed at all on this piece
2/Left flank	Delayed depilation	Heavy damage to whole piece only some small regions of grain surface remain	Heavy damage
2/Backbone-neck	Delayed depilation	Medium damage all over some very small regions of intact grain enamel immediately adjacent to residual wool	Completely eroded
2/Backbone-butt	Delayed depilation	Mild damage all over some small regions of intact grain enamel immediately adjacent to residual wool	Completely eroded
2/Right flank	Delayed depilation	Medium damage all over some very small regions of intact grain enamel immediately adjacent to residual wool	Completely eroded
2/Right flank	Immediately depilated	Medium damage all over some very small regions of intact grain enamel immediately adjacent to residual wool	Completely eroded

In addition to the skin pieces described in table 15, an additional sample was excised from the neck of skin one and treated using a conventional lime sulfide depilation process (as described in Appendix 8.2.1). This piece had undamaged grain indicating that the damage observed on the enzyme depilated pieces was solely associated with exposure to enzymes.

In the third experiment (Table 16) two skin pieces were exposed to enzyme solution (for 30 minutes) and then immediately treated with a conventional lime/sulfide depilatory without the holding period that the enzyme depilated pieces received. The intention of this work was to determine the damage to the skin caused by the initial exposure of the skins to the enzymes. These pieces, however, showed levels of damage to the grain that far exceeded those that had been wholly enzyme depilated. Entire portions of grain material were missing, leaving behind a ragged corium at the surface. This is in contrast to the excellent grain surface of the pieces that were not exposed to enzyme. Possible reasons for this observation include:

1. Immediate damage to the grain surface by the enzyme which was exacerbated by the action of the sulfide ions.
2. Modification of the activity of the enzyme by sulfide ions to increase its activity
3. Modification of the grain collagens by sulfide to make them more susceptible to damage by the enzyme.

It is known that sulfide breaks the disulfide cross links in proteins helping the denaturation process (O'Flaherty *et al.*, 1956). Due to the denaturing impact of sulfide ions on the enzyme for example it is unlikely that further enzyme activity produced this damage during the conventional processing step. In recent work (Allsop, 2007) has shown that sulfide depilation followed by enzyme processing in the place of the sulfide based liming process can be carried out with little or no damage to the skin. As such, modification of skin proteins by sulfide is probably not the cause of the damage observed here.

The first reason is therefore the most likely cause of damage; Removal or modification of components by the enzyme process causes the collagens in the skin to become susceptible to damage by sulfide ions. The nature of these modifications however is unknown at this time. In order to carry out subsequent processing using lime and sulfide the nature of these modifications would need to be identified, which is outside the scope of the work. It can be speculated that enzymatic hydrolysis of some proteins in the grain surface, not susceptible to the sulfide ions existent during conventional lime/sulfide depilation, may expose disulfide bonds within the grain surface that are integral to the integrity of the surface structure. In this way damage to the grain by subsequent alkaline sulfide processing may then occur (i.e. Enzymatic removal of specific proteins from the surface may enable the damage to the structures that maintain the surface character).

Over all, the results showed there was consistent damage to the pieces that depilated early, regardless of subsequent treatment. Evidence that wool sheath release does precede grain layer damage was observed however in skin pieces incorporating regions that were not depilated. These samples generally had small areas surrounding the non depilated region that were undamaged. The best pieces were those along the back bone that had barely depilated ie. depilated regions on the backbone adjacent to un-depilated regions. Typical examples of this band of undamaged region, fully damaged regions

located far from the non-depilation portion and conventionally depilated skin are illustrated in Figure 36, Figure 37, and Figure 38 respectively.

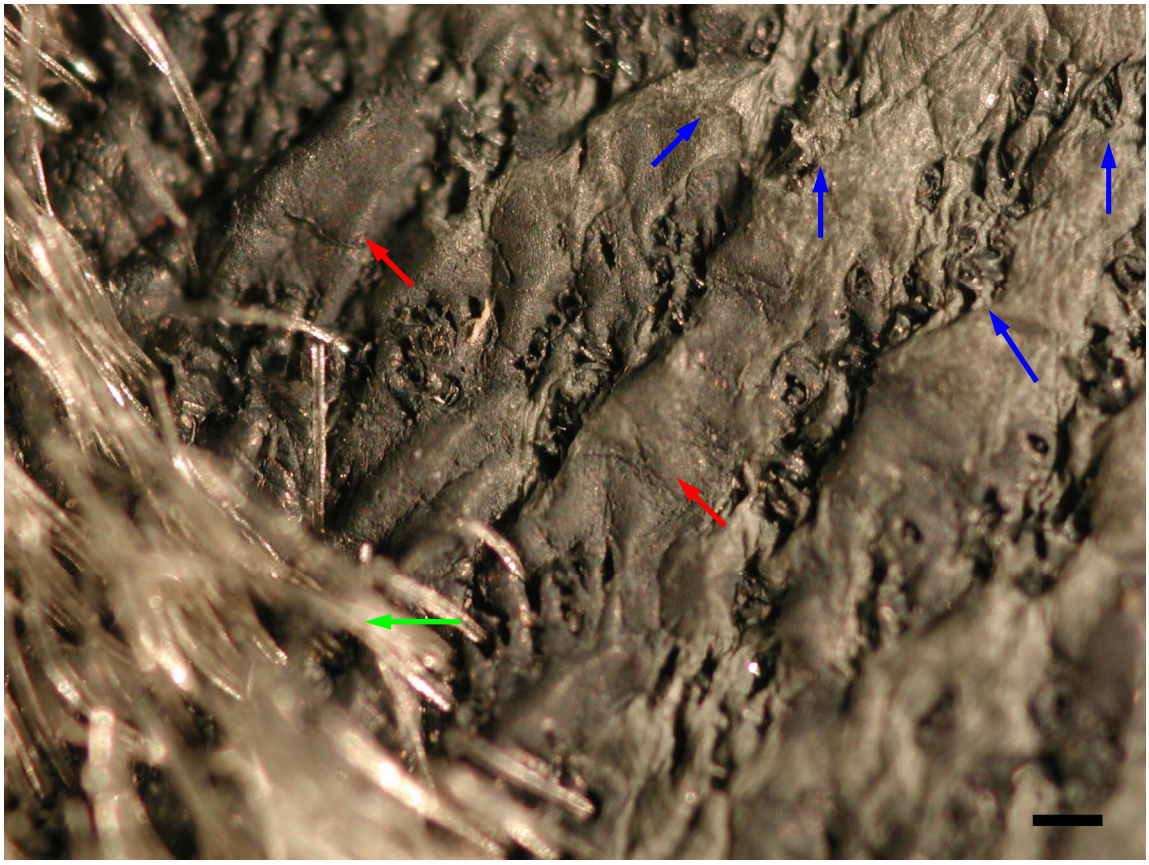


Figure 36: Region of undamaged skin surface (red arrows) located between non-depilated skin (green arrow) and damaged skin (blue arrows) (bar is 200 um). Damage becomes progressively worse the further away from the non-depilated region (not shown)

The band of undamaged skin (Figure 36) was measured on the pieces of skin from the final replicate of experiment above. It was found to be 1.50mm ± 0.16mm (95% confidence). This indicates that some (but not all) of the damage caused to the surface during the enzyme depilation process can be reduced by halting the enzyme activity just as depilation is achieved. The thickness of the band of undamaged skin between the non-depilated and damaged areas is related to the time at which depilation can occur before damage occurs. Hence if this time can be made as large as the width of the sigmoid curve (as shown in Figure 34), a successful enzymatic depilation could be carried out with this enzyme. The total area of the skins for that experiment were 0.75 and 1.1m² for each of the duplicate skins respectively.

Figure 37: Typical damage observed on the surface of enzyme depilated skins at a distance at least 5 cm away from the non-depilated regions (bar is 200 um).

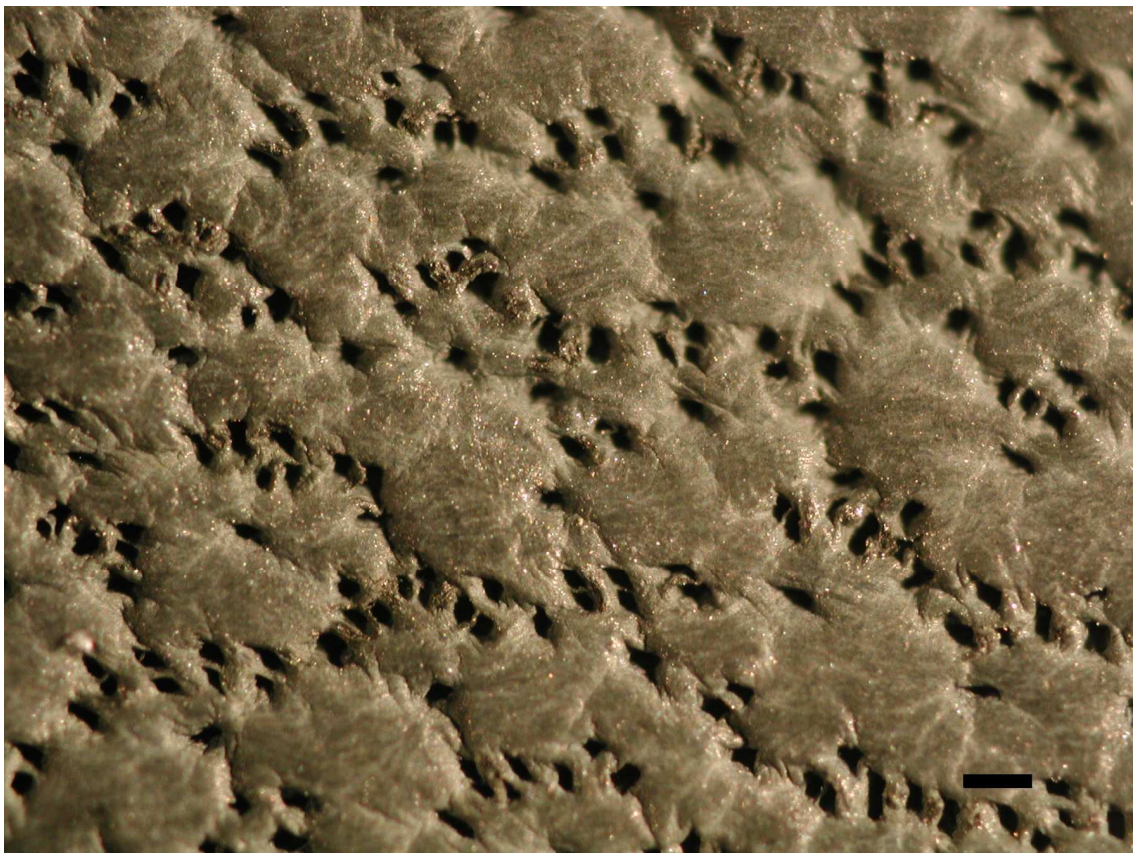


Figure 38: Conventionally processed skin from the neck region (bar is 200 um).

4.3 Variability of the skin (Skin heterogeneity)

Results so far indicate a difference in depilation and damage times across the skin, although the difference between depilation times and dewooling at any specific location is much smaller than the difference between depilation times across the skin. The backbone took longer than the flanks, even though the enzyme was evenly applied to the whole skin in an immersion process. The causes of this variation are likely to be due to variations in skin structure or composition across the pelt. An examination of the cause of this variation was therefore useful in terms of describing the enzyme depilation variation.

4.3.1 Approach

To allow comparison with the variability of skin properties across the skin, a quantitative measure of depilation progress across the skin as a function of time was required. This was achieved by recording the progression of depilation observed in section 4.2.1.1.

A template was constructed so that each of the parameters examined could be tested at the same location. A template was prepared to locate 41 sites on each skin (Figure 39). Quantification of the depilation process was carried out by recording the time taken to depilate the skin at each of these 41 sites.

The 41 scattered data points were interpolated into a regular 2 dimensional grid for the purpose of graphing. This was carried out using a Delaunay triangulation scheme to generate the regular grid and bi-cubic interpolation to interpolate the data points (MATLAB, 2004).

The results for time to depilate were averaged for the two skins and are illustrated in Figure 40.

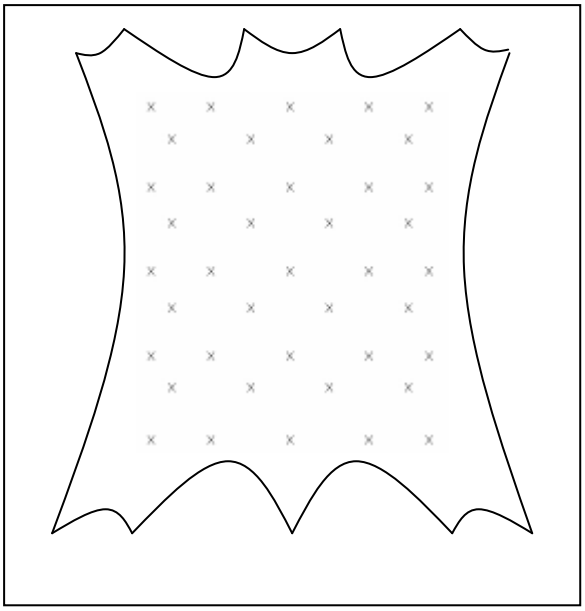


Figure 39: Sample sites. The relative location of each of the 41 sample positions are marked with an X.

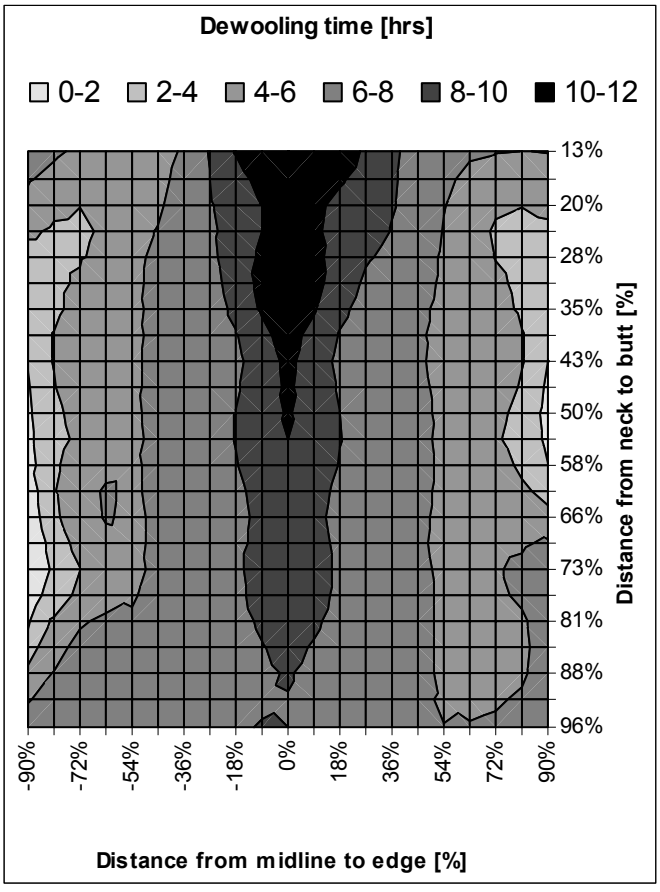


Figure 40: Enzyme depilation time. Enzyme depilation was shown to take longer down the mid line of the pelt, in particular in the neck area (top). The first depilation occurred in the middle of the flanks

The depilation showed a clear pattern beginning in the flanks after only 1 or 2 hours then continuing over the next 10 hours to progress towards the backbone and neck. The region of the skin taking the longest to depilate was the neck; along the backbone in particular. For each time step the area was measured by counting the pixels of the depilated regions. At the point at which each of the duplicate skins reached 50% depilation The perimeter of the depilated region was also measured. The rate of depilation at the point each skin reached 50% depilated was estimated from Figure 34. By multiplying the measured perimeter by the width of undamaged skin surface estimated in section 4.2.2, the area of the undamaged depilated region and the time taken to depilate that region can be calculated. Details of the depilation rate are given in Table 17.

Table 17: Rate of enzymatic depilation progression

Skin No.	Skin area [m²]	Rate of depilation at 50% depilated [%.hr^{-1}]	Rate of depilation at 50% depilated [m².hr^{-1}]	Perimeter of depilated region	Area of undamaged region at 50% depilated [m²]	Time to depilate good region only [min]
1	0.75	25 %.hr^{-1}	0.19 m².hr^{-1}	7.1 m	0.01 m²	3 min
2	1.00	21%.hr^{-1}	0.21 m².hr^{-1}	7.4 m	0.01 m²	2.9 min

Since the whole depilation takes about 8 hours depending on the individual skin, the window of opportunity to depilate a skin with no subsequent damage is quite small (8 hours compared to 3 minutes). From this it can be concluded that considerable reduction in the variation of depilation would be required to achieve a successful depilation using the enzyme system trialled so far.

4.3.2 Variable properties

In order to determine what properties of the skin might cause the observed variation in depilation rate, the heterogeneity across two skins of a number of properties was examined. For each property examined, the data is presented using the same contour presentation as Figure 40 for comparison of the shape of the distribution. Additionally a

plot of the correlation between depilation time and the property of interest is shown along side.

The skins depilated by enzymes in section 4.2.1 were processed through to crust leather and the properties examined. After 16 hours of total enzyme exposure time, the skins were pickled in the standard LASRA pickle with the addition of 2% formic acid at 10x dilution at the time when the sulfuric acid was added. The formic acid was used to neutralise the carbonate buffer. The pickled skins were then processed to crust leather using the standard LASRA process (See Appendix sections 8.2.5, 8.2.4, and 8.2.6 for full methodology). With the exception that all temperatures were kept below 30°C and no surfactant were added so that the grease content of the skin would not be mobilised and its distribution could be assessed.

4.3.2.1 Skin structure variation

Published work on the variation of ovine leather has shown similar variations across the skin for a number of physical properties. Stiffness, as measured by bending length and percentage extension tests have shown the skin to be stiffer in the region close to the backbone (Lange *et al.*, 2002). The variation in tensile strength is well known (Eskolin *et al.*, 1993). Tensile strength is higher in a direction parallel to the backbone rather than perpendicular to it. Tensile strength also decreases the further away from the backbone it is measured.

Although the variations of a number of structural factors have been investigated for ovine skin and are well described. The quantitative relationship between variable factors and the variability of enzymatic depilation has not been investigated. In this work each of a number of factors will be compared to the variation of enzymatic depilation. A simple correlation coefficient will be calculated for each to determine whether any correlation can be observed that will indicate a direction of investigation leading to understanding the general cause of variation in enzymatic depilation.

The variable fibre structure of sheep skins across the pelt has been previously described (Haines, 1981). It was shown that fibres predominantly lay along the lines of growth of the animal. That is, as the animal grows the skin grows with it particularly around the belly and along the back bone with collagen fibres being laid down predominantly in a direction parallel to the direction of growth. Specifically the collagen fibres in skin form a mesh in which the majority of fibres lie pointing away from the centre backbone line (Haines, 1981). In addition to the direction of fibres, the overall density of fibres also differs depending on their location across the skin. The fibre weaves of the butt and backbone areas are denser, and more closely packed in comparison to the regions of the flanks, with the regions of the axillae being the least dense of all (Haines, 1981),(Haines, 2006).

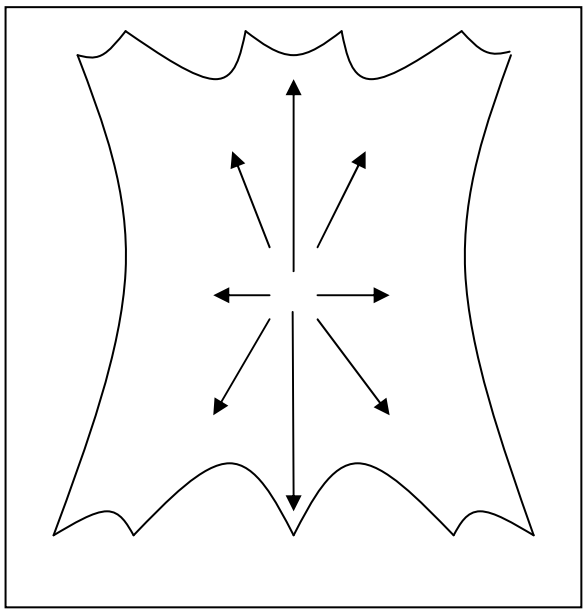


Figure 41: Fibre direction, adapted from Haines (2006).

The regions where fibre structure was most dense were also the regions most difficult to depilate. These regions were described as having the "corium fibre bundles interwoven compactly at a medium to high angle relative to the grain" (Haines, 1981) pg 14.

The angle of weave in the regions that are difficult to depilate by enzyme is interesting. A “high” angle of weave would result in the enzyme passing through the skin in a mostly parallel direction relative to the fibres. A low angle of weave, as found in the regions that are easy to depilate, would result in the enzyme passing through the skin in a direction perpendicular to the predominant direction of the collagen fibres. This is counter intuitive to conventional diffusion theory in which passage of material is fastest in a direction parallel to the fibre direction due to the shorter distances involved. This also suggests however that diffusion through the collagen fibre network is likely to vary across the skin due to these structural differences.

Data was available in the literature describing the stiffness of leather (Lange *et al.*, 2002). This data was presented in the literature both in terms of “bending length” and “extension” where increased bending length corresponds to stiffer leather and decreased extension corresponds to increased stiffness. These data were analysed using the interpolation technique described above to generate graphical representations of the stiffness properties of leather and the way in which they correlated to the variation in enzymatic depilation.

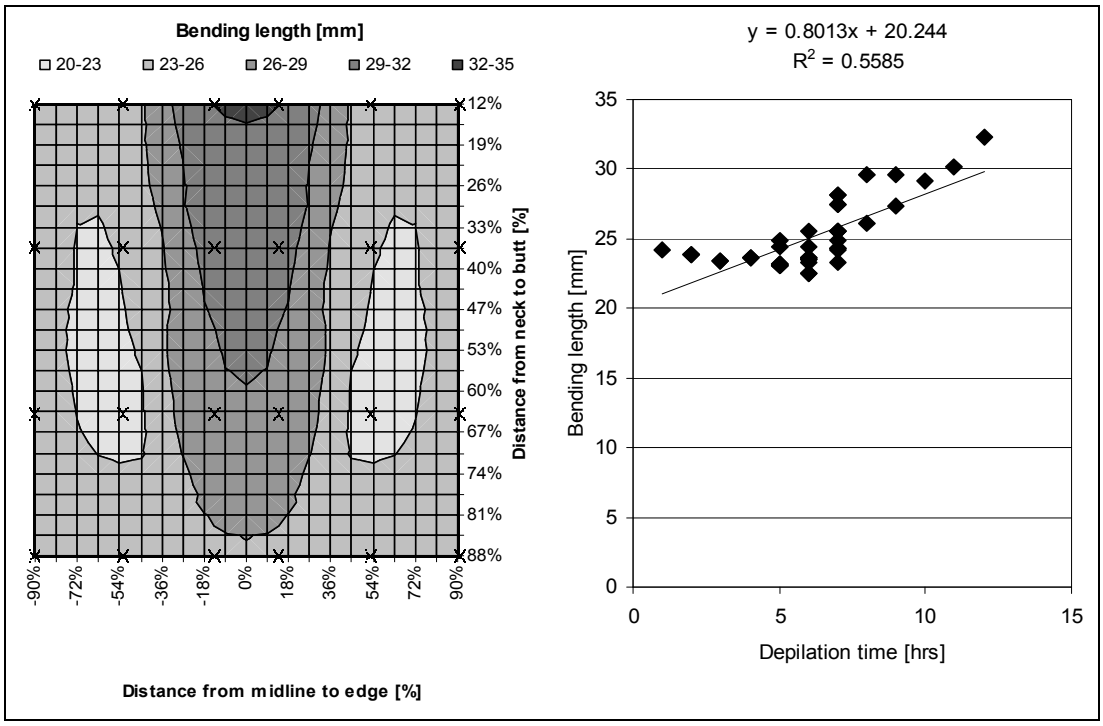


Figure 42: Left: Stiffness as measured by bendability, adapted from Lange *et al*, (2002). Sample positions are marked with an x. Note stiffness in the backbone. Right: Correlation of bending stiffness with enzyme depilation time. Note the good correlation p<0.001. The confirmation confirmation of a possible curvilinear relationship is left for future researchers.

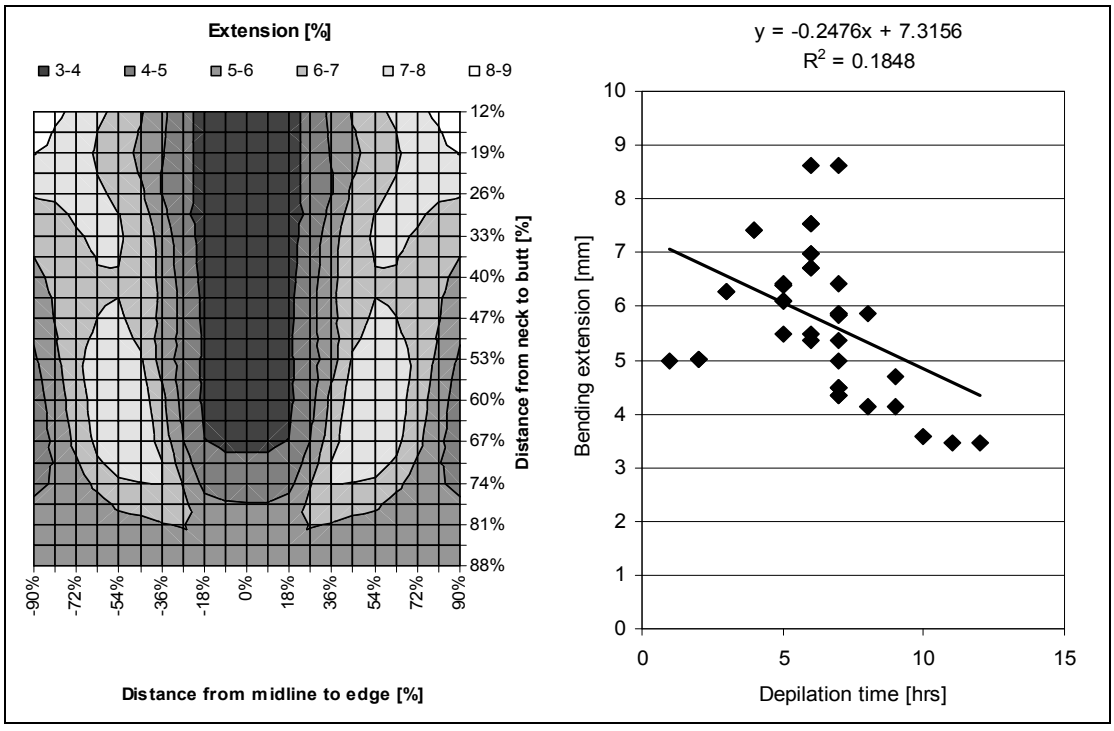


Figure 43: Left: Stiffness as measured by percent extension, adapted from Lange *et al*, (2002). Note the greater stiffness found towards the midline and less stiffness found in the flanks. Right: Correlation of bending stiffness with enzyme depilation time. Note the correlation (p=0.004).

The results show that stiffness as measured by bending length and extension show a good correlation with the variation observed for enzymatic depilation (Extension % r^2=0.18, p=0.004, bending length r^2=0.56 p<0.001). Reduced stiffness in the flanks and increased stiffness along the backbone and particularly in the neck, correlate well with the variation of enzymatic depilation. Stiffness is likely to be related to both the thickness of a skin (discussed below) and the collagen fibre structure. It can be speculated that a more dense collagen fibre structure would result in both an increase in stiffness and reduced rate of enzyme diffusion. Since collagen is insoluble, a greater concentration of collagen would result in a more tortuous path for the enzyme to diffuse through. Due to the inherent nature in the skin and final product, it is unlikely that the collagen distribution can be modified in a way to even out the enzymatic depilation process.

4.3.2.2 Variation in thickness

The most well known factor varying across skins is the thickness. Even after prefleshing, as in this example, the skin has a thickness profile that is related to the breed (Cooper, 1998b), but follows the same general pattern. To determine if the thickness was the cause of variation in the depilation rate of skins treated by immersion in the enzyme depilatory, the thickness of the crust leather was measured.

Thickness of the crust leather at each of the 41 sites was measured using an “Otto” substance gauge. In addition to the overall thickness of the crust leather, the thickness of the grain and corium were measured by examination of the appropriate cross section under the microscope. The results are illustrated in Figure 44.

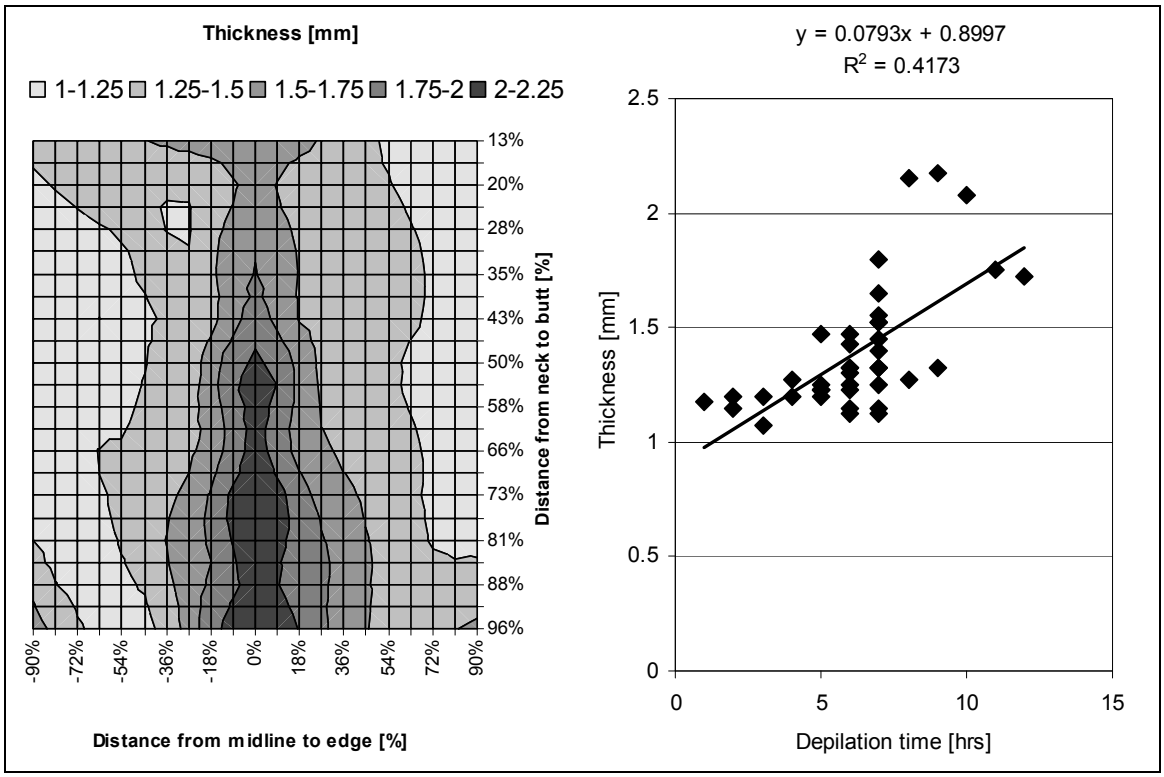


Figure 44: Left: Determination of the crust leather thickness. Note the thicker region down the back bone and in the butt area. This also corresponds to the same general pattern of thickness noted by previous authors (Cooper, 1998b). Right: Correlation of thickness with enzyme depilation time, note good correlation ($p<0.001$)

The skin thickness was correlated with the depilation time ($r^2=0.42$ $p<0.001$) but it is clear that this thickness difference does not offer a complete explanation for the pattern of the rate of depilation. The butt area was much thicker than the neck which was not the observed pattern of depilation in which the neck was the last region to depilate. So, while it may seem logical that skin thickness contributes to heterogeneous depilation it is not the only reason.

The total thickness of a lambskin is made up of the thickness of the grain and the thickness of the corium, and it is possible that one of these layers is important in the enzymatic depilation process. For this reason the crust leathers were sampled at each of the sites and the thicknesses of the grain and corium were measured under the microscope. The thickness of the grain and of the corium are illustrated in Figure 45 and Figure 46.

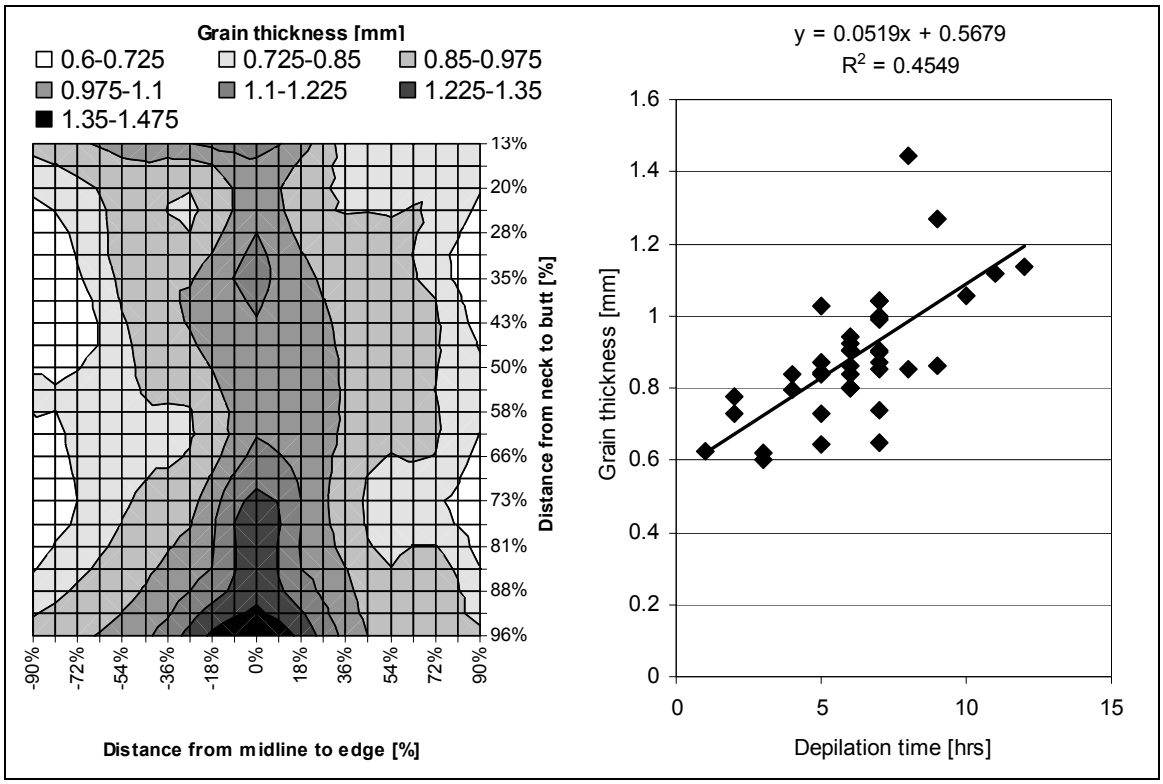


Figure 45: Left: Distribution of the grain thickness. Note the thicker grain in the butt region. Right: Correlation of grain thickness to enzyme depilation time, Note the good correlation (p<0.001)

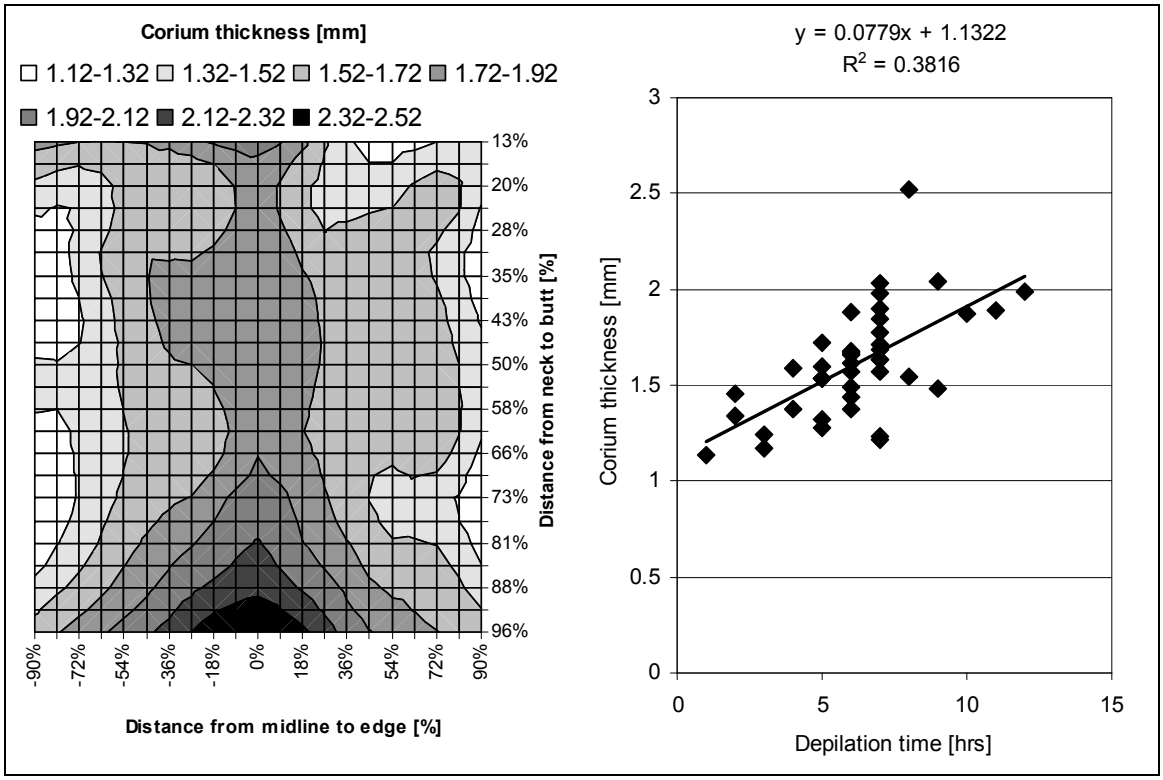


Figure 46: Left: Distribution of the corium thickness. Note the thicker grain in the butt region. Right: Correlation of corium thickness to enzyme depilation time, Note the good correlation (p<0.001)

In general the results for the thickness of the grain and corium follow the overall thickness profile as expected, with thicker grain and corium in the butt and along the backbone. Unsurprisingly both the grain and the corium thickness correlate with the variation in enzymatic depilation (grain $r^2=0.45$, corium $r^2=0.38$, $p<0.001$).

The wool roots are generally located at the base of the grain layer (see Figure 5). In an immersion application of enzyme the enzyme needs to pass through either the grain material or the corium material. Since the thicknesses for both grain and corium are highly correlated it is not possible to determine whether either is more important in terms of being a barrier to enzyme penetration. The ratio of the grain thickness to the corium thickness did not show any correlation to the enzymatic depilation variation ($p>0.1$) results not shown)

4.3.2.3 Grease content

Since the enzymes used in this work were water soluble, grease may act as a barrier to diffusion. The grease content of the skin was measured by processing the skin to crust leather without using surfactants or fatliquours and maintaining a temperature below 30°C so that the fat would not move during processing.

The actual measurement of grease was carried out in two ways. Firstly to assess the grease content of the full skin thickness, the standard method for grease determination ((SLC 4 ; IUC 4; Determination of Substances (Fats and Other Solubles) Soluble in Dichloromethane, 1996)) was used. Briefly, a sample of leather from each of the regions investigated was cut into pieces <1mm and subjected to Soxhlet extraction using dichloromethane for 5 hours at 6 cycles per hour. The amount of dichloromethane extractable material was then determined gravimetrically. The results for grease analysis across the full skin thickness are illustrated in Figure 47 below.

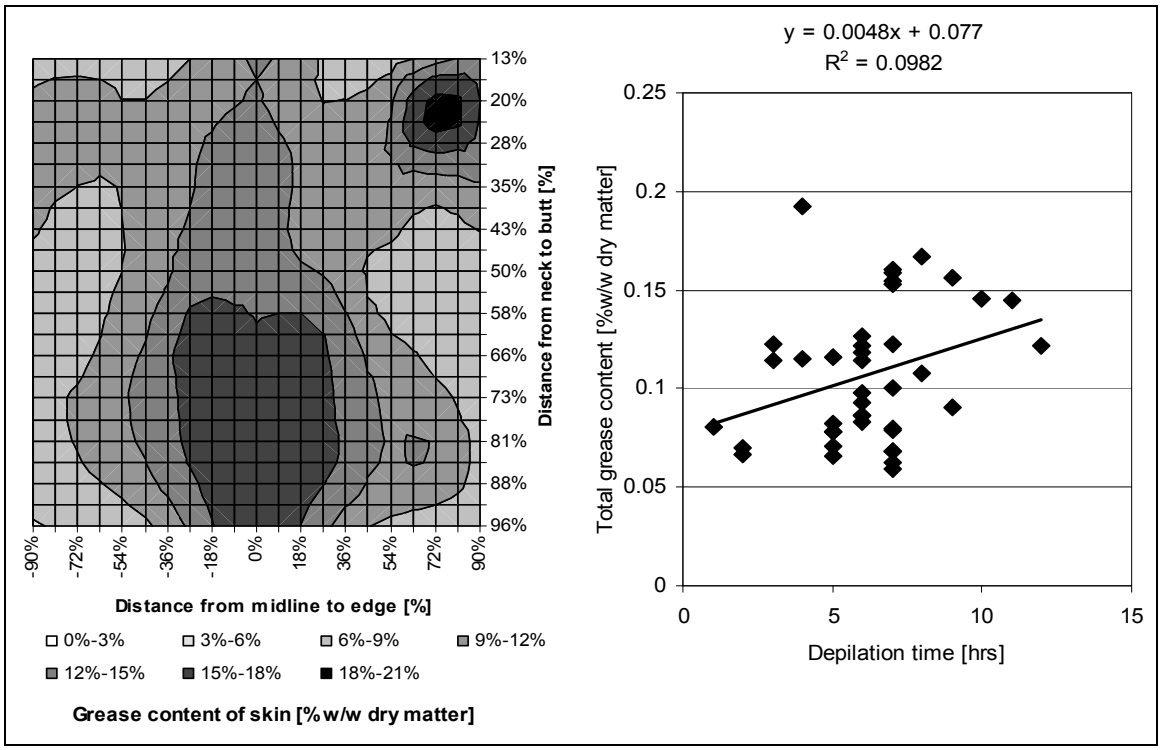


Figure 47: Left: Grease content of the full thickness of skin. Note the greater quantity of grease located in the midline and butt region. Right: Correlation of total grease to enzyme depilation time, note their was little correlation between the total grease and the depilation time indicating that the fat within the structure of the skin does not effect the depilation (p=0.04).

The dark region of Figure 47 in the top right portion indicates a high localised measurement of fat in the skin but it is likely that this result was due to an adherence of fat on the flesh surface and could be attributed to inadequate prefleshing. It is worth noting however that there are often regions located in the corner of the flanks which are poorly depilated (as can be seen in Figure 35 and the correlation of the fat and the location of those regions of poor depilation may not be coincidental. It may be possible that the presence of large quantities of fat present on the flesh side may inhibit the penetration of the enzyme depilatory specifically from the flesh side.

The results show there is a larger quantity of grease located along the back bone and in the butt regions of the pelt. The results follow work done previously on grease distribution of raw New Zealand lamb skins (Allsop & McClelland G., 1999) in which the distribution of grease through the full skin thickness was also found to be predominantly located in the neck and along the back bone. These data correlate only weakly with the enzymatic depilation variation ($p=0.04$, $r^2=0.10$) indicating that the

majority of grease within the skin does not strongly affect the penetration and reaction of the enzyme.

When fat is unevenly distributed throughout the skin, particularly when sebaceous grease is not removed from the surface, it results in an uneven dying with darker regions in areas with greater levels of grease (Pauckner & Herfeld, 1968), (Test and Research Institute for Shoe Manufacturing, 1976). To monitor the distribution of grease on the surface of the skin in this trial, the crust leathers were assessed for their colour brightness (C-lab L value) using a Datacolor International microflash 200d. By directly assessing the colour variability the relative content of grease at the surface can therefore be estimated.

The grease content at the surface of the skin was therefore determined based on the observation that the presence of grease at the surface of the leather results in darker patches. The skins were processed below the melting point of fat and without any surfactants so that the grease contained in the surface would remain in the same regions and be visible as darker regions resulting in measurable brightness differences in the crust leather.

The results for the surface grease content as determined by the surface darkness are illustrated in Figure 48.

The darkness results were well correlated with the depilation results (r^2=0.45, $p<0.001$). It is possible therefore that grease forms a less penetrable physical barrier to the enzyme solution inhibiting its access through the skin to the wool root. The presence of wool grease within the follicles may act as a direct barrier for the enzyme to access the cells of the root sheath. This is in contrast to the skin grease located within the skin structure which occurs within fat cells located predominantly in the grain-corium junction and do not appear to be correlated with the variation of enzymatic depilation.

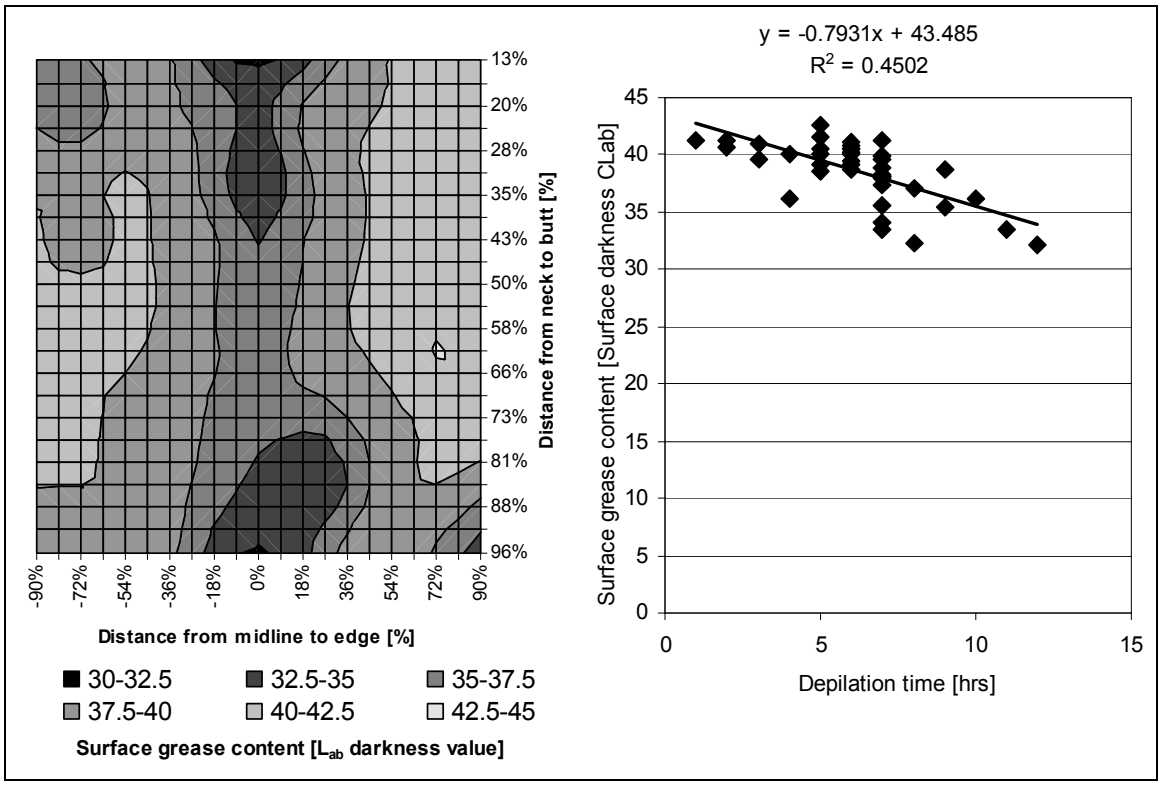


Figure 48: Left: Grease content in the skin surface as measured by surface darkness. Note the darker regions at the mid line of the neck and butt. Right: Correlation of surface grease content with depilation time, note the good correlation ($p<0.001$) nb: darker regions are denoted by lower values for L_{ab}

The slight correlation between the grease as measured over the full thickness of the pelt and the enzymatic depilation variation may be explained by the presence of wool grease which does correlate with the enzymatic depilation variation. The use of surfactants or solvents to improve enzyme unhairing processes have previously been suggested (Cantera *et al.*, 2005). It may therefore be useful to incorporate surfactants or solvents that target the sebaceous grease into the immersion enzyme system to achieve a more even depilation result.

4.3.2.4 Fibre diameter

The force required to remove a wool fibre is a function of the strength by which it is held in the wool root. The greater the fibre diameter the greater the surface area of contact between the wool root and the rest of the skin matrix. It follows therefore that the rate of depilation may be related to the fibre diameter.

Wool fibres (12 or so) were manually removed from the skin, examined under the microscope and the fibre diameter of each wool fibre was measured directly. Twelve wool fibres were examined from each test and the results averaged. Samples of wool were taken from each of the 41 sites and the fibre diameter measured microscopically (Figure 49).

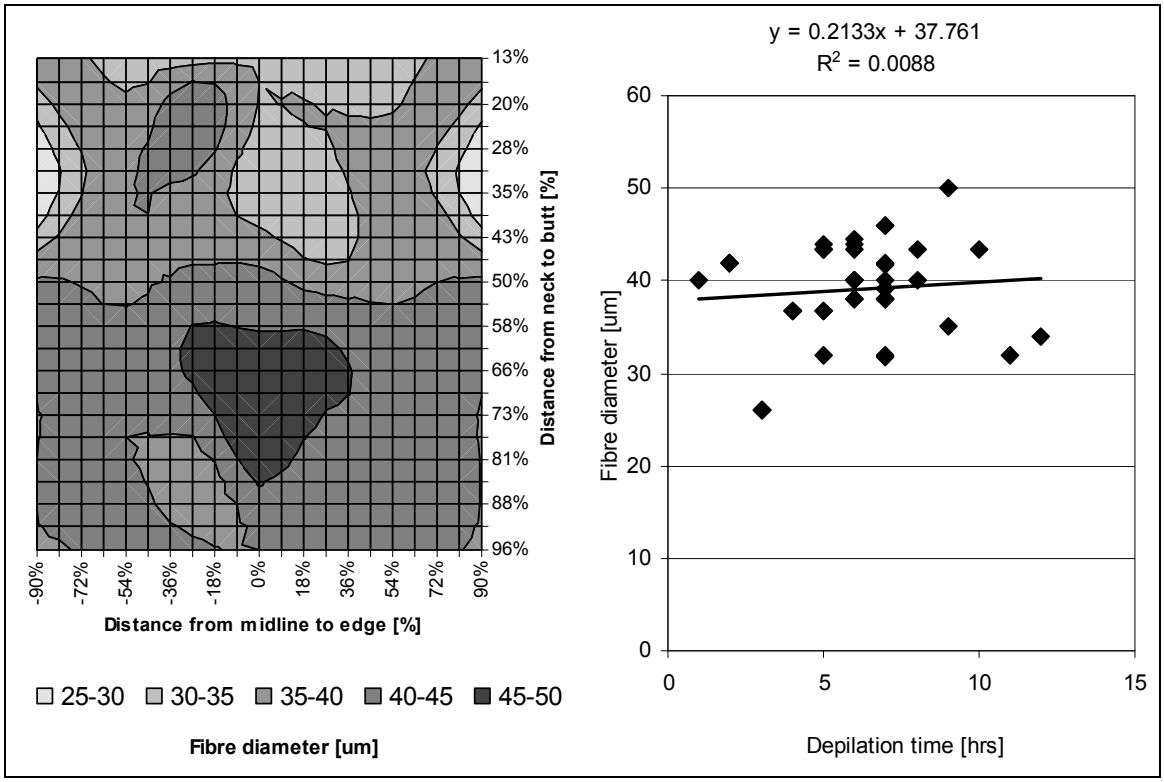


Figure 49: Left: Distribution of fibre diameter. Note the greater fibre diameter in the lower middle of the back. Right correlation of fibre diameter to enzyme depilation time, note the lack of correlation (p>0.1)

The fibre diameter was only slightly variable across the skin, the largest fibres being found in the middle of the butt area. The fibre diameter was not found to be related to the rate of enzyme depilation (r^2 =0.009, p>0. 1), indicating that the rate at which enzyme depilation occurred was not related to the size of the fibre. This suggests it is time for the enzyme to reach the wool fibre root that limits depilation rather than the time taken to remove material around the wool fibre in order to loosen it.

4.3.2.5 Follicle distribution

Intuitively it is likely to be more difficult to pull a large number of wool fibres from the skin together than a few. Thus those areas of the skin with a denser distribution of wool follicles may take longer to depilate. If the force applied to the skin to depilate the wool is spread over relatively more fibres then this will have an impact on the ability for that region to be depilated at a given point of wool loosening. Furthermore, if the depilation reaction is limited by the ratio of enzyme concentration to the number of wool follicles then a region of the pelt containing a larger number of wool follicles would be exposed to less enzyme per wool fibre and take longer to depilate.

A method to determine the follicle density distribution of ovine skin on live animals using photographs and computer analysis has been previously developed (Nagorcka *et al.*, 1995). In this work the distribution was simply determined by directly assessing the final crust leather and counting the wool shaft tunnels under the microscope. Each of the 41 sites on the crust leather was examined under the microscope and every follicle canal in a 0.5 by 0.5 cm area was counted.

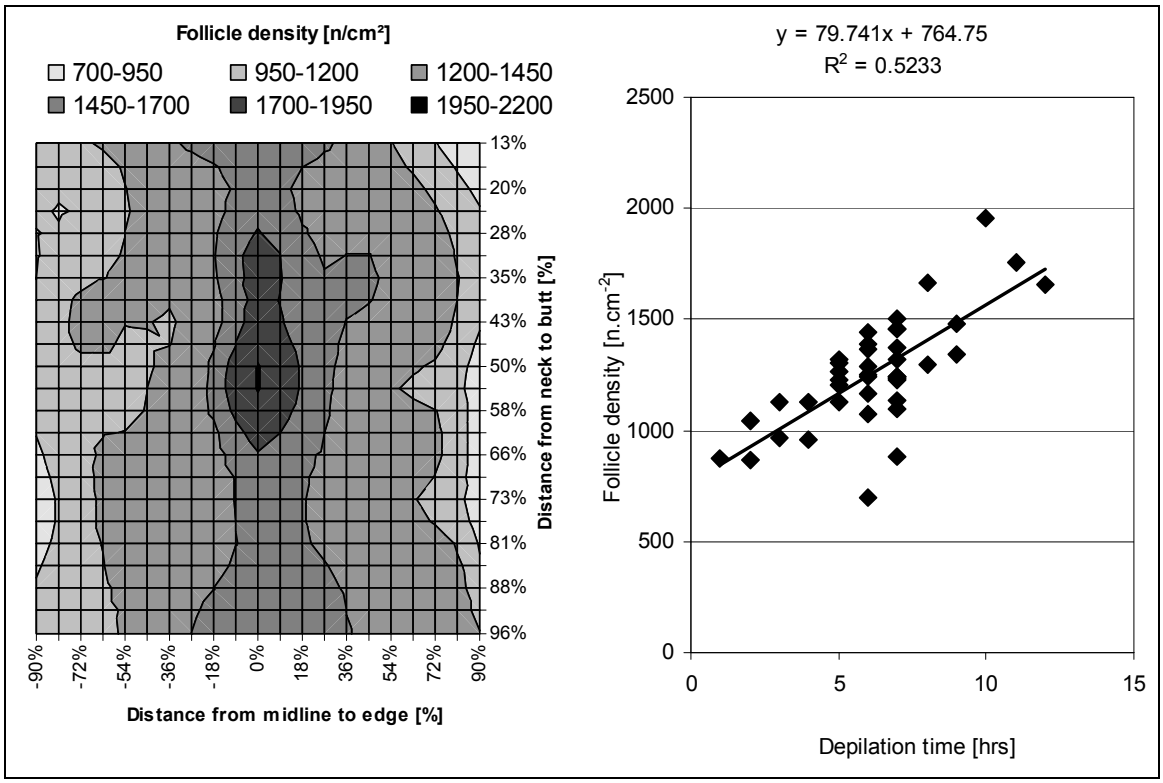


Figure 50: Left: Distribution of follicle density. Note the greater follicle density in the centre of the back. Correlation of follicle density to enzymatic depilation time, note the good correlation ($p<0.001$).

The density of the follicles found on the skins examined in this experiment is illustrated in Figure 50. The results show that the highest concentration of follicles was along the backbone. The results measured here for New Zealand Romney lambskins is in subtle contrast to those previously described for Merino lambskins (D'Arcy, 1979). While Merino skins do have an increased level of follicles in the centre of the back, they also have regions of higher follicle density in the butt and in the neck (D'Arcy, 1979). Overall the data correlates reasonably well to the depilation pattern observed on the Romney skins, with an increased depilation time along the backbone (r^2 =0.52, $p<0.001$) but is less well correlated to the increased depilation time required in regions towards the neck relative to the butt. While it may seem logical that follicle density is a reason for heterogeneity in depilation effectiveness, it is not the only reason.

4.3.2.6 Wool root strength

For a similar reason as described above for follicle density, the wool root strength could explain why some areas of the skin require longer processing before depilation is possible.

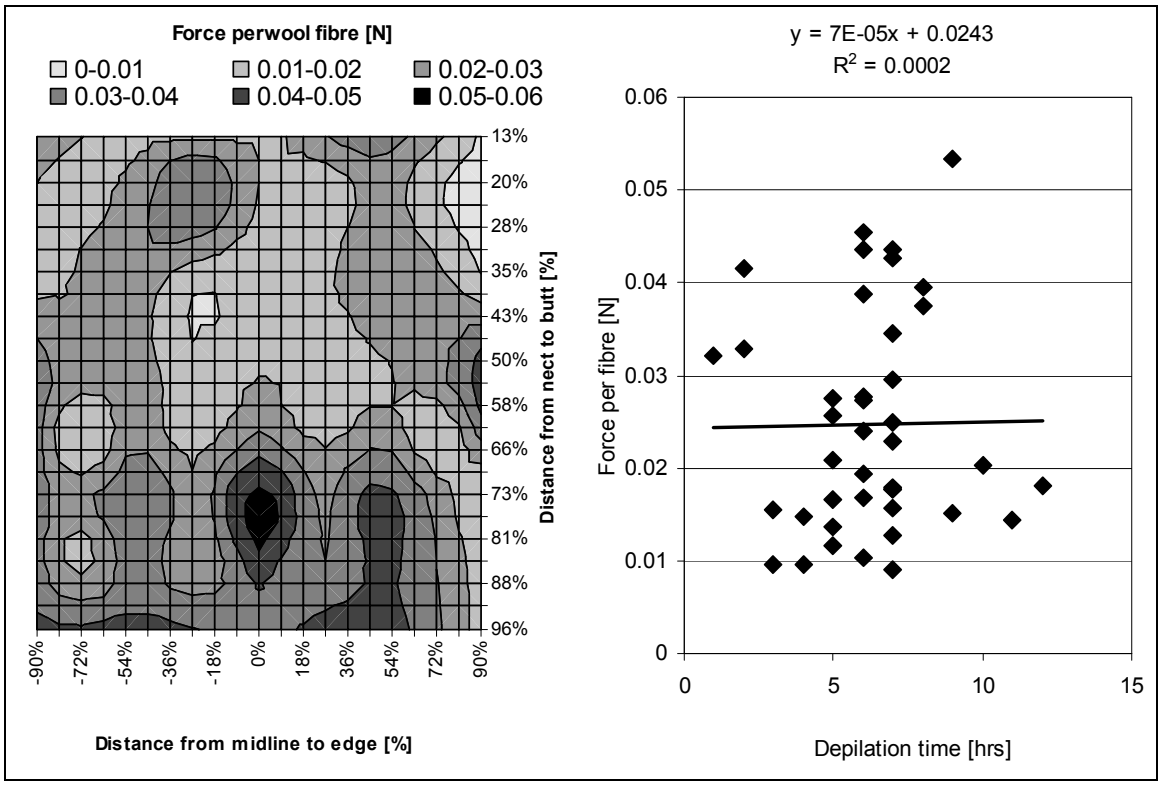


Figure 51: Left: Force per wool fibre. Note a slight increase in force per fibre in the lower middle of the back. Right : Correlation of force per wool fibre with enzymatic depilation time, note the lack of correlation (p>0.1).

The strength by which individual wool fibres are held within the wool root sheath of the raw skin was measured using the Lennox test (Yates, 1964). Briefly; a staple of wool from each region was attached to a force gauge and the force in Newtons required to pull the staple from the skin was measured. The number of wool fibres in each staple was then counted and the measured force normalised against this number. The results are illustrated in Figure 51.

The results showed a slight increase in the force required to pull individual fibres in the region towards the centre of the butt. The data therefore shows that there is a difference in the way fibres are held in the skin in this region but there was no correlation with the enzyme depilation variation (r^2=0.0002,p>0.1). There was a definite similarity between the pattern of wool root strength noted here and the pattern of wool fibre diameter, thus indicating an interaction between the diameter of the wool fibre and the force required to pull each fibre out (see Figure 52). Thicker fibres are likely to be more difficult to remove from the raw skin simply because there is a greater area of contact between the skin and the fibre and potential for larger numbers of chemical bonds attaching it to the skin structure. It therefore follows that some of the pattern observed for the wool root strength observed here may be due to the larger diameter of the fibre. By plotting the force per wool fibre against the fibre diameter measurements at each of the sites this relationship becomes clear.

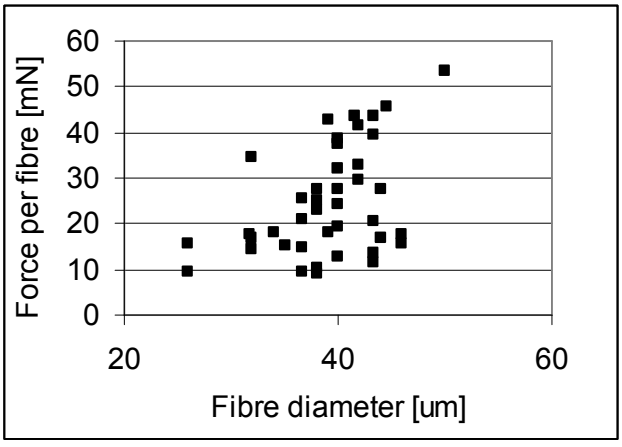


Figure 52: Relationship between the forces required to remove a wool fibre from a raw skin to the fibre diameter as measured at each of the 41 sites. There is a weak correlation between the force required to pull out a wool fibre and the diameter of that fibre (r^2 =0.20 p=0.003)

The results indicate that there is a relationship between how strongly the wool fibre is held in the skin and the fibre diameter. However, there does not appear to be any relationship between how well the fibres are held in the raw skin and the enzymatic depilation process.

4.3.3 Skin variation – Summary

Overall the best correlation between the pattern of depilation in an immersion application of enzyme depilatory and measured skin parameters is a combination of skin thickness, surface fat distribution, and follicle density. There did not appear to be any relationship between the depilation pattern and the force required to remove individual wool fibres from the raw skin. This indicates that the heterogeneity observed during enzyme depilation is related to physical aspects of the skin such as thickness and the presence of a grease barrier rather than chemical or biochemical aspects of the wool root and its depilation reaction mechanism. It was also evident that in areas of high follicle density, longer times were required for depilation. This is likely to be due to the requirement of weaker individual root bonding strength if more wool fibres are removed through application of the same force since a given force per unit area would be spread over more fibres in a region of higher fibre density. While this observation is interesting, and could explain some of the variations in depilation behaviour across the skin, it cannot be changed during processing. It is possible that a complete analysis of the data using multiple regression may lead to a more comprehensive quantitative relationship between each of the physical parameters which correlated to the depilation variation and this work should become the subject of future studies. Analysis at this stage indicates that further investigations in this work targeting more even enzyme depilation should therefore focus on homogenising those physical factors which affect the penetration and concentration of enzyme depilatory at the wool root, such as pre-fleshing to even out the skin thickness, the removal of materials from within the skin which act as barriers to depilatory reagents, and the removal of non collagen materials in the skin in the region down to the wool bulb that make up the thickness such as the soluble proteins or glycosaminoglycans.

For this reason the next portion of the work was an investigation on the effectiveness of washing skins prior to depilation to reduce the concentration of the substances which may act as a barrier to the enzyme solution.

4.4 Impact of washing on soluble proteins from the skin

Sheep and lamb skins contain a large number of components. Of primary interest are the insoluble collagens which become the pickled pelt and then leather, and wool fibre that is removed during fellmongering and is a valuable product in its own right. Most other substances are removed during the fellmongering process. These include water soluble proteins and glycosaminoglycans, which make up a large portion of the non-commercial substance within skin material. It is postulated that these materials provide a barrier to depilatory reagents, which indicates that the early removal of these water soluble materials may aid in the depilation process and result in a more evenly depilated product.

The aim of this experiment was to determine whether these water soluble materials could be removed from a raw skin by a washing regime and to observe what affect this had on enzyme depilation.

Initially a trial was carried out to demonstrate whether washing skins caused any measurable changes to the skin properties. Well washed skins and skins that were not washed at all were examined and specific characteristics such as changes in the ease of depilation and possible structural changes to the wool root were compared.

A more detailed examination of screen washing was then undertaken to determine the effect of different screen wash durations on the ease of depilation. Screen washing is a common system used in the New Zealand industry.

4.4.1 Initial washing proof-of-concept trial

In order to determine if washing had any measurable effect on depilation an initial trial was carried out using a protracted washing regime.

4.4.1.1 Method

A matched pair trial was conducted with one half of each skin receiving a protracted washing regime and the other half of each skin being held in a chilled state. Samples were then analysed for changes in structure.

For the experiment, 34 skins were obtained from a cooperating meat processing plant and chilled to 0°C using ice. Each skin was then split down the back bone. The left side of each skin was held in ice at 0°C until painting, while the right hand side of each skin was treated by a washing regime that was more rigorous than any used in industry. Skins were immersed in a 20x volume of water at 35°C for 16 hours containing 0.5% (w/v float concentration) potassium dimethyl-dithiocarbamate to prevent bacterial growth. Half skins were divided into two groups and washed in two research processing vessels (Dose drums, 450L capacity) by rotation at 8 rpm for 5 minutes per hour for a total of 16 hours. The skins were then chilled to 15 °C. The control skins which had been held overnight at 0°C were taken out of the ice and allowed to warm to the ambient temperature of 15°C. At this time, samples were taken from the skins for analysis and microscopic evaluation of the wool root structures.

The skins were analysed for both soluble protein, and total protein ((SLC 7; IUC 10; BS 1309:7; Determination of Nitrogen and Hide Substance, 1996)). Briefly, for total protein quadruplicate samples from the washed skins were cut into small pieces (<2mm). Approximately 2g of skin sample was then treated to Kjeldahl digestion, and protein determination was carried out as follows; The entire sample was weighed into a 500mL Kjeldahl flask containing approximately 5g of Kjeldahl catalyst (9.1% w/w anhydrous $CuSO_4$, 90.9% anhydrous K_2SO_4. Concentrated sulfuric acid (20 mL) was then added and the mixture digested until clear (~30 min). After cooling the sample, 150 mL water was added, and the flask connected to a Kjeldahl apparatus. A 500 mL conical flask containing 100 mL boric acid (40g/L boric acid) and10 mL boric acid indicator (0.3 g/L methyl red, 0.2g/L methylene blue in ethanol) was placed at the receiving condenser with the tube immersed in the boric acid solution. NaOH (75 mL of 40% w/v solution) was added to the flask and the contents heated for about 15 minutes until the ammonia had been driven off and the solution “bumped”. The distillate was then titrated against 0.1250 M sulfuric acid until the indicator turned violet.

For soluble protein, quadruplicate 10g skin samples were cut into small pieces (<2mm) and placed in a 1 litre capacity glass jar with 4.2 g anhydrous sodium carbonate and 500 mL water. The skin samples were then shaken intermittently for one hour then incubated without shaking for 1 hour this cycle was repeated over a total of 24 hours. 250 mL of liquid from the glass jars were then filtered (Whatman No.54) and added to Kjeldahl flasks containing 5 g of Kjeldahl catalyst and 20 mL concentrated sulfuric acid. The samples were then treated to Kjeldahl digestion and protein determined as described above.

The skins were also sampled and examined histologically using the SACPIC stain (section 8.3.3), which specifically highlights the skin structures. Four of the skins were then depilated using the enzyme depilation technique described in section 3.2.1.2 with the exception that only 2% enzyme was used instead of 4%, to determine if there had been any improvement in the effectiveness of depilation. The remainder were processed through to crust leather using standard processes (section 8.1) in order to examine the effect of washing on the physical properties of the leather. Standard techniques were used to investigate the effect of washing on the properties of the leather for three reasons; so that the impact of washing could be more easily assessed by comparison to conventional leather; to ensure that washing itself had no adverse effects on the leather surface; and to assess if any bacterial damage had occurred.

4.4.1.2 Results

4.4.1.2.1 Changes in depilation from washing

The skins were examined for their level of depilation and the results are illustrated in Figure 53. The results showed a significant improvement in depilation when skins were washed. Skins that were to be processed by conventional means were also assessed for the effect of washing on the conventional lime-sulfide depilation. Skin washing was found to give a significant improvement on conventional depilation also (results not shown).

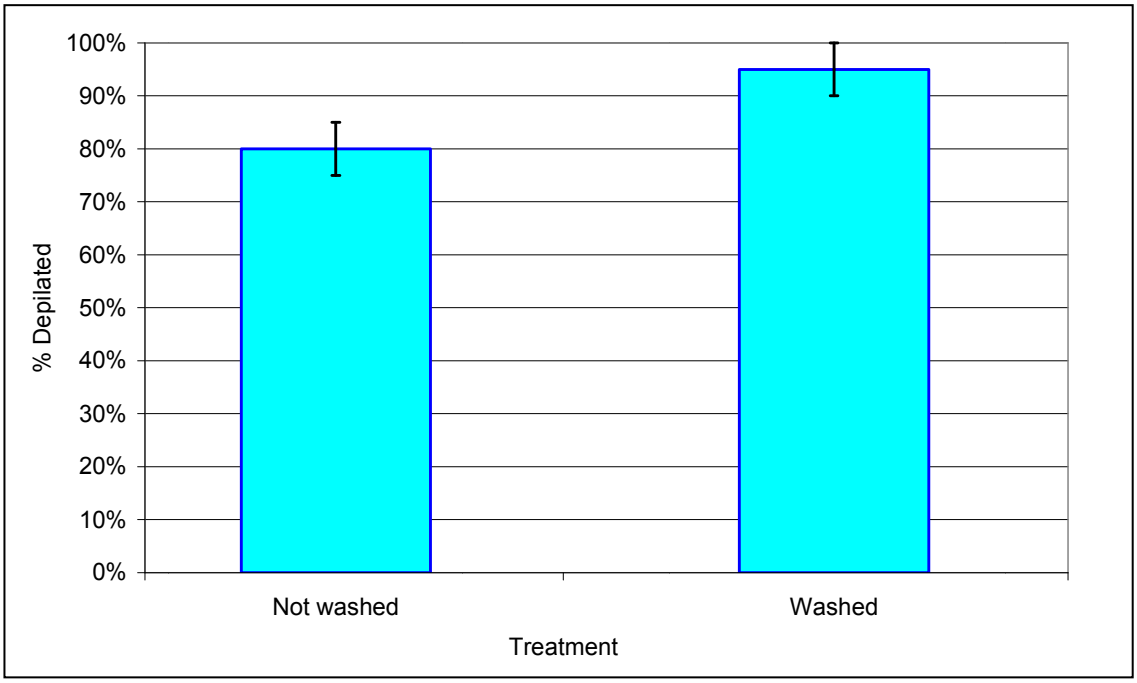


Figure 53: Comparison of the extent of enzymatic depilation after 16 hours from washed vs. unwashed skins. Note the increase in the level of depilation when skins are washed.

Specifically, wool was easier to remove and less wool remained on the fringes. Every skin was compared to its matched half after depilation and in each case the skin which had received the washing regime had less residual wool than its unwashed counterpart. It is also important to note that the skin that had been washed had less residual wool in the neck region which is known to be resistant to wool removal. The washing, therefore resulted in the general depilatory effectiveness becoming slightly more even. These observations are significant because they indicate a difference in the structure of the raw material due to the washing.

After depilation the skins were drummed in an 80% float for liming as per the standard method. Significant differences were observed in the liming liquor. More wool was visible in the lime liquor from the unwashed skins with the float from the washed skins appearing more fluid with less wool.

An increase in depilatory effectiveness occurred for the conventional lime-sulfide process which validates the improved effectiveness observed for enzymatic depilation. It seems therefore that, as expected, the removal of water soluble materials improves the effectiveness of a “depilatory”. Removal of material from regions that contain relatively

more soluble material is therefore a reasonable mode for improving the uniformity of depilation.

4.4.1.2.2 Skin structure

Samples of the raw skins were taken for microscopic examination and the structure of the follicles examined employing a SACPIC stain. Examination of the sections indicated that there was no effect on the structures of the skin from the washing process. The epidermis remained intact, the wool fibre was not damaged, both the inner and outer root sheaths remained intact, and the wool root bulb also was not affected. No differences in structure were evident between the washed skin and the unwashed (results not shown).

4.4.1.2.3 Protein removal

The soluble protein and total protein results are presented in Figure 54 as a percentage of the total skin dry matter and as a percentage of the total protein.

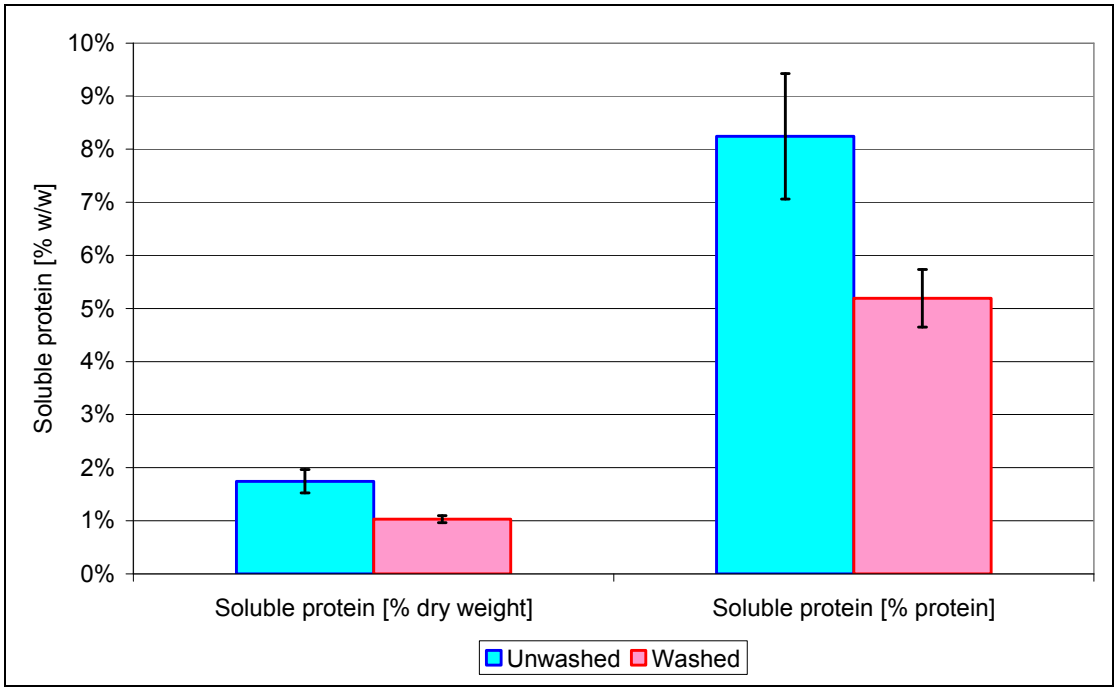


Figure 54: Soluble protein removed during washing. Note that both the absolute amount of soluble protein and the proportion of protein that was soluble decreased after washing.

The results show that washing the skins had a significant effect on the level of soluble protein in the skin. In this experiment, close to half of the soluble protein was removed during washing, and this removal of protein affected depilation. The ratio of soluble

protein to total protein shows that, as expected, more of the protein that remained after washing was insoluble, indicating that washing did not affect the structural proteins in the skins such as the collagens. It is likely that equilibrium between soluble protein in the skin and float was achieved due to the long time frame used in the washing step. As such a second washing stage would remove more of this soluble material and further increase depilation uniformity.

It was found that there was no significant difference in the levels of soluble protein in the pickled pelts between the skins that were washed and those which were not. The changes in soluble protein found in the skins after washing did not therefore effect the composition of the final product.

The removal of soluble protein from the skins during washing would have a profound effect on the penetration of depilatory reagents. Protein provides a buffering capacity to the skin which needs to be overcome by the presence of caustic before depilation with alkaline proteases can occur. In addition, a broad spectrum protease will react with much of the protein it comes in contact with during penetration, regardless of its source. By removing the soluble proteins, a potential substrate is removed from the depilation system thereby improving the efficacy of the depilatory reagent. The removal of soluble protein by efficient washing should therefore enhance the overall depilation process by improving both the penetration and efficiency of the depilatory enzyme. By improving the washing that is carried out during the cooling process which typically happens immediately after the skin is taken off the animal, it would be possible to achieve a number of these advantages without substantially changing the current system.

4.4.1.2.4 Crust leather

The pickled pelts were processed through to dyed crust leather using standard LASRA processes (section 8.1) and then tested for physical properties likely to be effected by changes in the protein content and depilation process such as looseness, softness, grain appearance and strength. Standard processes were used for these comparisons so that the impact of the washing could be assessed and the results would not be confounded by the known damaging effects of enzyme depilation.

4.4.1.2.4.1 Looseness

The looseness was measured in duplicate at five locations on each skin; neck, upper mid, mid, lower mid, and butt at a position 1/3 of the distance from the centre of the backbone to the edge of the flanks. The results were then averaged for each of the different experimental sets. The score system ranged from 1 representing very tight leather to 8 which represented very loose leather (Lowe & Cooper, 1998). Briefly, the crust lamb leather is folded so that creases can be seen. The creases are then compared with a subjective scale of increasing looseness. Skin with no visible wrinkles when the skin is folded are scored 1 and are considered tight. Leather with the largest wrinkles are scored 8 and are considered loose. The results are illustrated in Figure 55.

The results indicated that even extreme washing had no effect on the looseness of the final crust leather.

Figure 55: Impact of washing on looseness

4.4.1.2.4.2 Softness

The softness was measured in duplicate at five locations on each skin; neck, upper mid, mid, lower mid, and butt. The method for measuring softness was the standard method (IUP 36, 2000). Briefly, The leather is clamped in a specialised device and a probe pressed into the leather. The depth to which the probe is able to be pressed is then

recorded in mm. The results were averaged for each of the different experimental sets and are illustrated in Figure 56.

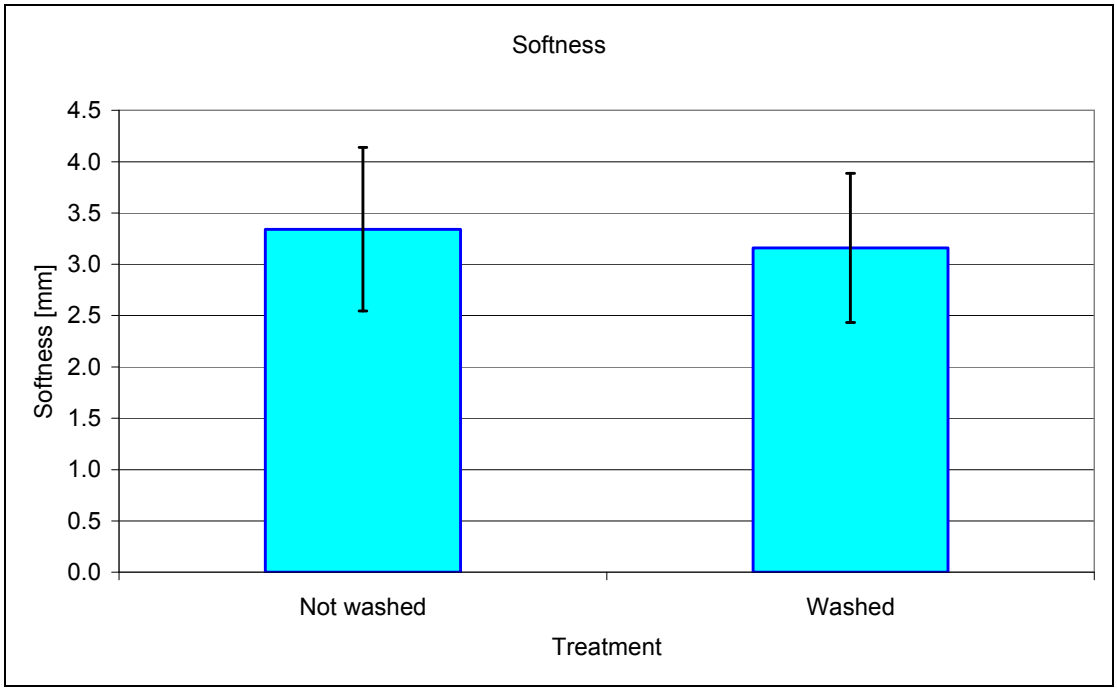


Figure 56: The impact of washing on the softness of crust leather

A greater value indicates softer leather. The results indicated that there was no significant difference between the groups of skins that were washed compared with the skins that were not. This further supports the likelihood that the manner in which skins are washed before processing had little impact on the quality of the final leather.

4.4.1.2.4.3 Grain surface

A bacteriostat was added during the washing process to stop any bacterial damage and the success of this was assessed by examination of the crust leather after conventional possessing. The surfaces of the skins were also assessed after conventional processing through to crust to ensure that the washing process did not introduce any new form of damage to the surface.

The grain surface for each piece was examined under an optical microscope at four different sites. The quality of the grain was compared to a subjective scale as follows:

Table 18: Grain surface assessment descriptive scale.

Surface assessment	Subjective score
5	No free collagen fibres visible at all – No distortion of the grain
4	A few loose collagen fibres visible – Some distortion of the grain
3	Some loose collagen fibres visible – Some grain destroyed
2	Many loose collagen fibres visible – Much of the grain destroyed
1	Grain surface completely destroyed

The results (illustrated in Figure 57) show that again, there was no difference in the quality of the grain surface between the skins that received the washing treatment and those that did not. This confirms that the quality of the crust leather was not adversely affected by the washing treatment.

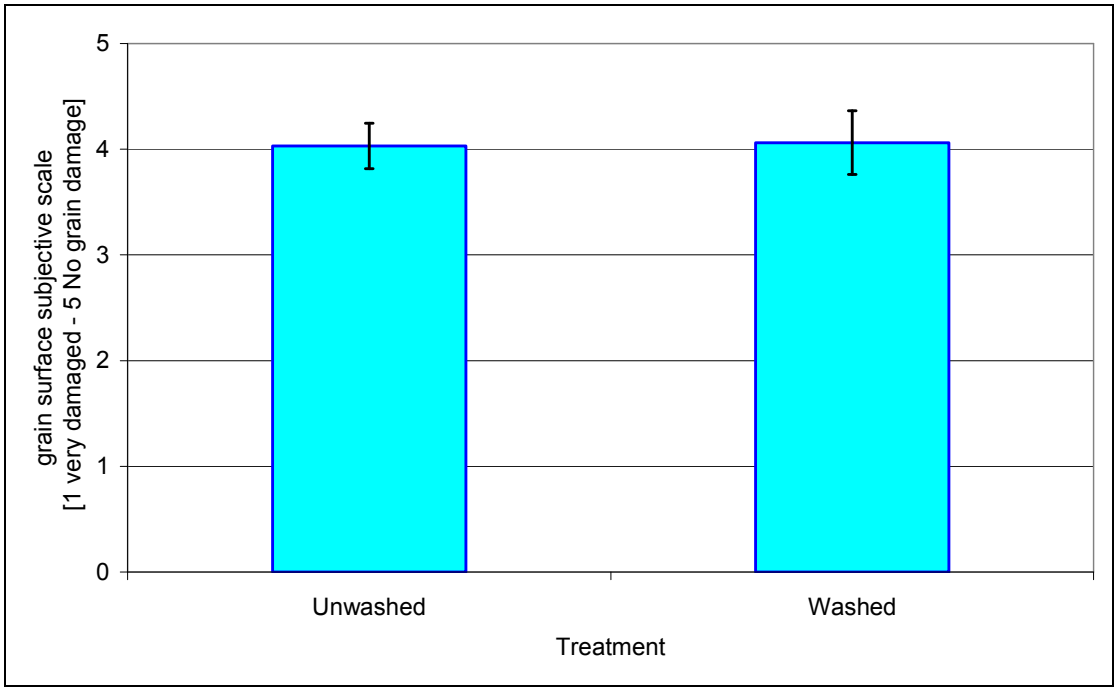


Figure 57: Effect of washing on the quality of the grain surface

4.4.1.2.4.4 Strength of leather after washing

Both the tensile and tear strengths of the crust leathers were tested using the standard methods (IUP 6, 2000) (IUP 8, 2000). Briefly, samples were cut from the leather at the official sampling positions (IUP 2, 2000). The samples were then conditioned by holding at a constant temperature and humidity (20°C and 65% relative humidity) for 24 hours, after which time the samples were tested on an Instron testing device. The results are illustrated in Figure 58. These results show that the strength of the crust leather was unaffected by even the extreme washing process.

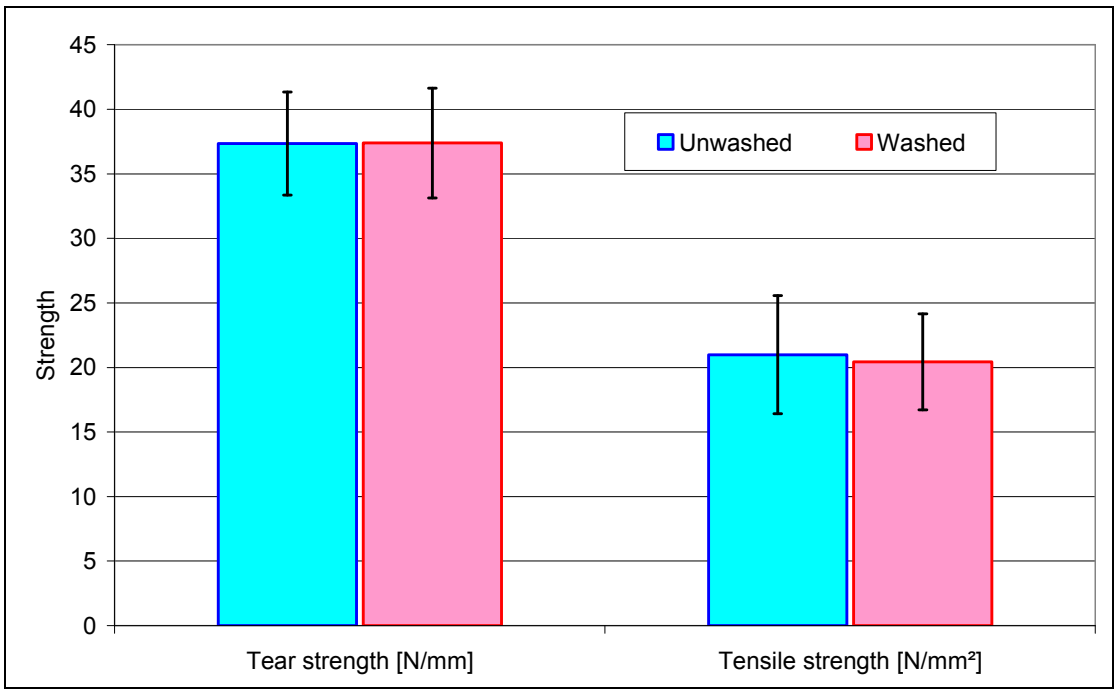


Figure 58: Impact of washing on the strength of crust leather

The results indicated that significant benefits in terms of improved depilation could be gained from effective washing of skins prior to application of the depilatory. The crust leather product generated from skins with the extreme washing regime was as tight and soft as the control. The grain surface was also found to be undamaged and the strength of the leather from each group was the same. This indicates that provided the skins are protected from bacterial degradation as they were in this experiment, rigorous washing of the skins prior to depilation by either lime sulfide or enzymes, has significant benefit for the process.

4.4.2 Detailed washing experiment

In order to determine the exact nature and quantity of improvement in enzyme depilation effectiveness through the use of washing a further experiment was carried out as follows.

4.4.2.1 Method

Fresh skins were collected from a cooperating pelt processing plant and were prefleshed and trimmed to remove meat and fat adhering to the flesh surface. The skins were then cut into matched quarters. Skins were washed at 30°C in two volumes of water containing 1% of a bacteriostat potassium dimethyldithiocarbamate solution (Busan 85, Buckmans: Australia). Skins were washed for 0,4,8, or 16 hours. After washing the skins were soaked in volumes of enzyme depilatory solution containing: 1% Purafect 4000L (v/v), 0.5 M Sodium carbonate/bicarbonate buffer at pH 10.5, 0.5% potassium dimethyldithiocarbamate solution (Busan 85, Buckmans: Australia) - 30%w/v active, and 0.5% Teric BL8 (Huntsman: Australia). After 30 minutes the skins were squeezed dry using a mangle to remove excess depilatory and the amount of wool loosened assessed at 4 hour intervals by the method described in section 3.2.1.2 Results are illustrated in Figure 59.

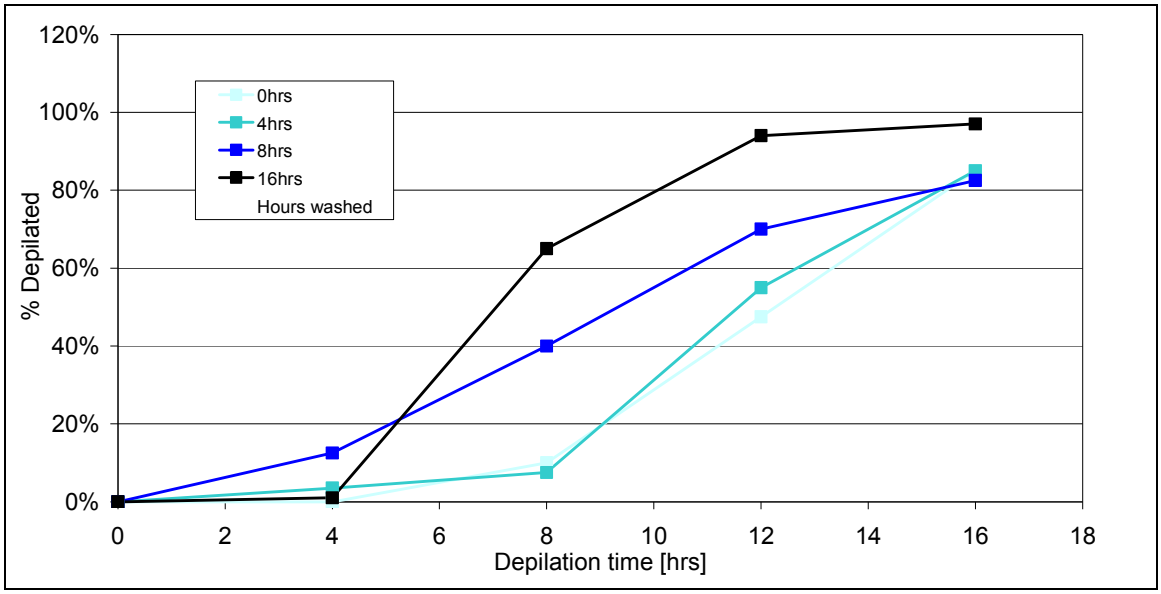


Figure 59: The effect of washing on the progress of enzyme depilation. Different colours represent different washing times, The x-axis represents the time after depilatory enzyme exposure. Note the improvement in the rate of depilation for skins that were exposed to washing for longer periods.

Washing the skins had a clear effect on the rate at which the skin was depilated. The longer the skin was washed for the more quickly it became depilated. It is likely that washing the skins removed some soluble material from the skin allowing the enzyme to easily penetrate the skin. The sigmoid shaped curve describing the progression of depilation as described in section 4.2.1 was again found when observing the effects of washing the skins. Unfortunately the improvement in the rate of depilation did not change the overall shape of the sigmoid curve. The time for the whole skin to become mostly depilated after the onset of depilation in the flank regions was about the same in each case. The shape of the sigmoid curve was not made more steep to give more even depilation. Rather, the whole curve was shifted to the left resulting in a similar reduction in the depilation time for each region. These results suggest that washing will strongly influence depilation time, but will not overcome the issues regarding uneven depilation and the onset of damage of the enamel grain layer.

4.4.3 Removal of soluble proteins - Summary

Prolonged skin washing was carried out to determine whether it had any effect on the depilation process. It was found that washing the skins improved the depilation for both enzymatically and conventionally depilated skin. Investigation of the washed skins showed that there was no visible change in either the wool root or wool shaft, suggesting that a chemical or compositional change was the reason for improved depilation. Measurement of both total and soluble proteins remaining in the skins after washing showed that soluble protein levels were reduced in the skins that were washed.

For the washed skins that were processed through to crust leather using conventional methods washing appeared to have no effect on the protein composition of the pickled pelts. In addition the quality of the final crust leather was the same as for those skins that had not been washed. This indicated that a significant extension of the washing process could give improvements to the depilation process without adversely affecting the product.

4.5 Variability of enzyme depilation – Conclusions

The aim of this chapter was to characterise the variation in depilation in comparison to the natural skin variation, this aim was successfully achieved with the following results.

An investigation of within skin variation indicated that the variation observed during enzyme depilation was related to physical properties of the skin rather than chemical or biochemical aspects of the wool root and that removal of barriers to penetration would be likely to improve the evenness of depilation

Washing skins removed soluble proteins and improved the rate of depilation but did not significantly improve the evenness of depilation.

Other methods of improving the penetration of enzyme to the depilatory site could be investigated including the use of solvent to remove fats or the removal of glycosaminoglycans, but if they respond the same way as the removal of soluble proteins then it is likely that the risk to the skin of over exposure to the enzyme will remain since a considerable improvement in the evenness of depilation would be required.

Instead a more profitable direction would be an investigation into the mechanism of the damage causing process itself. This is investigated in the following chapters.

Component requirements for enzymatic depilation

5.1 Introduction

In the previous chapter it was shown that general protease damage-free dewooling across the skin is unlikely by simply reducing the variability across the skin. The relatively small time difference between wool sheath loosening and grain layer damage, mean a more specific activity is required either to loosen the wool, or to avoid enzyme activity on the structures that must remain to prevent damage. An investigation into the molecular basis of the damage to the skin structure incurred during enzymatic depilation was therefore carried out. At the same time, more specific methods of enzyme depilation were tried in order to attempt to find an enzyme or enzymes that could be used to achieve dewooling without the accompanying damage to the skin surface.

5.2 The structural mechanism of enzyme depilation

The literature showed that the most important targets for effective depilation are the cellular sheaths surrounding the hair-root and the complex structures that attach those root sheaths to the rest of the skin. The aim of this section was to confirm the importance of the structures in the skin in terms of the enzymatic depilation mechanism. The integrity of the skin structures were probed during the enzyme depilation process using a modified version of a Safranine – Celestin blue – Picro-indigo-carmine (SACPIC) follicle staining technique (Yates, 1968a) section 8.3.3. This stain differentiates the major structures involved in the dewooling process such as the inner and outer root sheath as well as the surrounding collagen. The assay was carried out to investigate the changes in those structures during enzyme depilation.

5.2.1 Initial investigation of the enzyme depilation mechanism

In order to confirm the mechanism for enzyme depilation in general, the effects of the complete set of enzymes obtained in section 3.2 using the enzymes described in Table 5 were examined. Skin samples for depilation were prepared as described in section 3.2.1.2 Samples were taken at 4,8,16, and 20 hours after the initial exposure to the

enzyme solution and fixed in buffered formalin solution (see section 7.2.1.2). Samples taken from near (10cm) the backbone were cryogenically frozen on a freezing microtome, sectioned, and stained with the "SACPIC" staining procedure (see appendix 8.3.)

5.2.2 Enzyme depilation mechanism – SACPIC results

Typical results of the SACPIC stain procedure are illustrated in Figure 60.

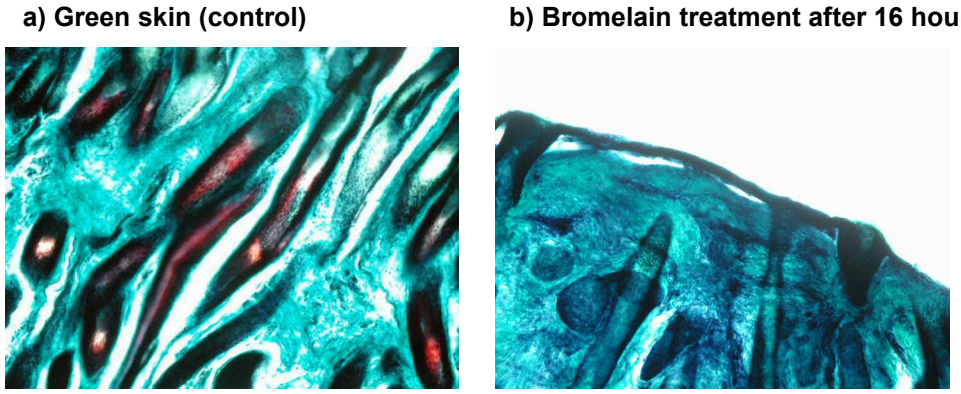


Figure 60: Typical SACPIC stains of cross sections of skin (each image has the same scale, bar is 50 um)

In the green skin control illustrated in Figure 60a the epidermis and the cells of the outer root sheath can be seen as the darker regions. When viewed under the microscope, individual cells are easily discerned. Although not visible in the photographs, the wool shafts were stained yellow and could be easily identified. The inner root sheath (IRS) was stained red. The stain penetrated and was fixed only when the IRS was intact and the outer root sheath (ORS) had been removed (as a consequence of section preparation). When the inner root sheath was not stained red it appeared similar to the ORS. Collagen was stained blue/green.

In the Bromelain treated skin (Figure 60b), the wool roots and epidermis are clearly visible, but the epidermis was separated from the grain layer. The histological changes apparent in SACPIC stained sections from skins treated with various enzymes are summarised in Table 19.

Table 19: Results of SACPIC staining during the enzymatic depilation process

Enzyme	4 hours	8 hours	16 hours	20 hours
Promod 278P	No damage observed.	Large regions of destruction to grain collagen and ORS.	Increasing levels of damage.	General break up of all structures observed.
Protease 6L	No damage observed.	ORS and IRS largely damaged; epidermis lifting and breaking up.	Most epidermis gone. ORS/IRS gone in some places. Grain layer damaged.	Little original structure remains. Most structure broken up. Some destruction of grain layer.
Protease 200	No damage observed.	Some splitting up of ORS and epidermis.	Slightly more break-up of ORS/IRS. Some lifting of epidermis.	Lower portion of ORS now amorphous. Upper ORS mostly intact. Only some epidermis lifting off.
Batinase Neutral	Small amount of breaking up of epidermis.	Small amount of ORS beginning to break-up.	Root sheaths largely broken up. Extensive lifting of epidermis.	Little original structure remains. Most structure broken up. Grain layer mostly intact.
Bromelain	No damage observed.	Large amounts of ORS beginning to break-up.	ORS and IRS largely damaged, epidermis breaking up.	Lower portion of ORS now amorphous. Upper ORS mostly intact. Epidermis and upper ORS lifting from grain surface.
Purafect 4000L	Small amount of ORS beginning to break-up.	ORS and IRS largely damaged; epidermis lifting.	Most epidermis gone. ORS/IRS gone in some places. Hairs separating from root sheaths.	Most root sheath amorphous. Some damage to grain surface.

Damage to the skin appeared to follow a similar pattern for all enzymes during the depilation process. Firstly the lower portion of the ORS was damaged. Next the ORS and epidermis lifted from the grain surface and finally the cellular structure of the ORS/IRS and epidermis breaks up. Promod was found to be effective in aiding the removal of wool but the staining technique showed that it caused damage to the collagen quite early in the process. Samples processed with Protease 6L showed early evidence of the ORS breaking up and lifting of the epidermis, which was followed by damage to the grain layer at a later time. Samples exposed to Protease 200 appeared to exhibit the same characteristics as those exposed to Protease 6L, although the extent of

degradation was less. Samples processed with Batinase Neutral showed very early breakdown of the epidermis along with extensive modification to the collagen structure.

Bromelain exhibited a relatively specific activity against the ORS and epidermis early on, but after 20 hours there was extensive lifting of the epidermis that was accompanied by the breakdown of the upper region of the ORS. However the efficiency of depilation was low compared to the other enzyme formulations, indicating that the lifting of the epidermis and ORS alone are not sufficient for complete depilation. Some other activity appears to be required in order to achieve good depilation. Purafect showed highly specific activity against the ORS/IRS and caused the epidermis to lift early. However towards the end of the depilation process damage to the grain layer began.

Overall the investigation of the depilation mechanism of the selected proteolytic enzymes is consistent with the earlier screening work which indicated that Purafect 4000L was the best performer since the depilation was best and destruction to the grain layer the least.

Many proteolytic enzymes can achieve depilation as shown in Table 19. However all the enzymes investigated in this study also damaged the grain enamel to a greater or lesser extent. The enzymes that were tested are industrial formulations that contain broad spectrum enzyme activities, some of which are probably unnecessary for the depilation process. These enzymes are formulated to meet requirements for wide spectrum proteolytic activity and are promoted for use in industries other than leather manufacturing such as food modification. They have not been optimised for depilation. The exact enzymatic requirements for good wool removal without damage to the underlying skin structure, particularly the collagen network therefore needs to be determined

5.2.3 Structural modification – Summary

Comparisons of the mechanism of depilation by different enzymes using the SACPIC stain technique indicated that all the enzymes investigated had similar effects in the skin structure. First, the lower portion of the outer root sheath was destroyed. Next the

epidermis and outer root sheath lifted from the grain layer and finally damage began to occur to the grain collagen.

Observations showed that the proteins involved in cementing the cells of the epidermis to the underlying matrix are potential targets for a refined enzyme depilation process, although this is not the only requirement for complete and efficient depilation. Further loosening of the hair from the follicle is also required which might be aided by destruction of cells in the root sheaths. Damage to the grain surface was visible after the point at which the structures around the wool root were destroyed.

This result confirms that the two pronged approach attempted in this work was sensible. Specifically a combination of making the depilation process itself more uniform to avoid the onset of damage due to excessive exposure of some parts of the pelt to the enzyme, combined with an investigation of the damaging activities of the enzyme.

5.3 Glycosaminoglycan changes during enzyme depilation

The literature review showed there were several different points of view about the role of glycosaminoglycans in enzyme depilation. Because they are a major component of the basement membrane, the removal of glycosaminoglycans would appear to be desirable for enzyme depilation. However, it was also noted that using specific glycosidases alone did not achieve depilation (Yates, 1972). Reports from the literature indicate that, although they are not specifically involved in holding wool into the skin matrix, the removal of glycosaminoglycans is likely to facilitate depilation because of easier access of depilatory agents to the wool bulb and root shaft. Due to the apparent importance of these skin components, experiments were carried out to characterise their role in the enzyme depilation mechanism.

5.3.1 Method

Although it is possible to determine the amount of sulfated glycosaminoglycans in skin using quantitative techniques (Mozersky *et al.*, 2003), an investigation of their histological location was more useful in this case as it would relate the effect of changes in the glycosaminoglycan content directly with the changes occurring in the depilatory

active region. Skin samples were taken from the depilation trials described in section 5.2 and fixed in buffered formalin solution (see section 7.2.1.2), at 4, 8, 16, and 20 hours after the initial exposure to the enzyme solution. The samples were frozen, and sectioned as before, and then stained with : Alcian blue / periodic acid Schiff (AB-PAS) Section 8.3.4 ((Walsh & Jass, 2000)).

5.3.2 Results

Representative photomicrographs of typical enzyme treated and untreated skins after staining are shown in Figure 61.

a) Green skin (control) **b) Treated with Protease 200 at 20 hr**

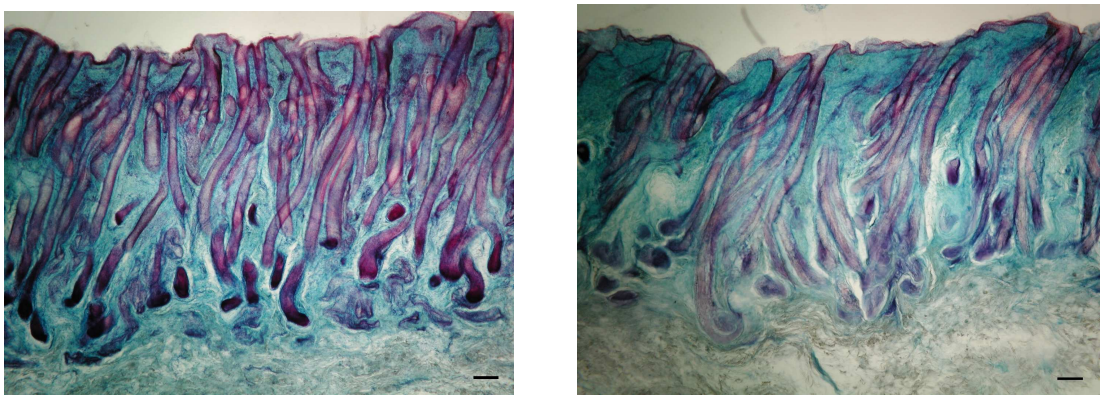


Figure 61: Alcian blue- Periodic acid Schiff staining of skin cross sections (both images are at the same scale, bar is 100 um) Where: regions stained blue contain carboxyl or sulfate groups, regions stained magenta contained material with glycol units such as sugars and regions stained purple contain both.

The AB - PAS results for the different enzymes explored are summarised in Table 20. In each section the corium appeared unstained indicating that little glycosaminoglycans were present in the corium. The wool shafts were also unstained again indicating a lack of glycosaminoglycans

Table 20: Alcian blue - Periodic acid Schiff staining results where regions stained blue contain carboxyl or sulfate groups, regions stained magenta contained material with glycol units such as sugars and regions stained purple contain both.

Name/time	Grain	Epidermis	Lower ORS	Upper ORS	Bulb
Green skin	Blue.	Purple.	Light purple.	Light purple.	Dark purple to black.
Bromelain 4 hours	Blue.	Purple.	Light purple.	Light purple.	Dark purple to black.
Bromelain 8 hours	Faded blue.	Purple, some sloughing.	Light purple some loss of definition.	Light purple some sloughing.	Light purple.
Bromelain 16 hours	Faded blue.	Purple, some sloughing.	Very light purple heavy loss of definition.	Light purple some sloughing.	Very light purple heavy loss of definition.
Bromelain 20 hours	Faded blue.	Purple, complete sloughing.	Very light purple heavy loss of definition.	Light purple some sloughing some loss of definition.	Very light purple heavy loss of definition.
Batinase 4 hours	blue (darker at grain surface).	Purple.	Light purple.	Light purple.	Dark purple.
Batinase 8 hours	blue (darker at grain surface).	Purple, some breaking up.	Light purple some loss of definition.	Light purple some breaking up no loss of definition.	Dark purple.
Batinase 16 hours	Blue (darker at grain surface).	Purple, heavy breaking up.	Very light purple heavy loss of definition.	Light purple, heavy breaking up coincident with epidermis.	Light purple some loss of definition.
Batinase 20 hours	Blue (darker at grain surface) some loss of definition.	Purple, mostly gone.	Very light purple heavy loss of definition. Mostly gone.	Almost all gone dark purple lifting with wool shafts.	Very light purple heavy loss of definition.
Protease 6L 4 hours	Green.	Purple.	Purple.	Purple.	Dark purple.
Protease 6L 8 hours	Faded green.	Purple, some breaking up.	Light purple some loss of definition.	Purple.	Light purple some loss of definition some breaking up.
Protease 6L 16 hours	blue (darker at grain surface).	Purple, some sloughing.	Light purple some breaking up.	Purple.	Light purple some loss of definition some breaking up.
Protease 6L 20 hours	Light blue.	Purple, heavy sloughing.	Mostly broken up some staining purple.	Unstained.	Light purple heavy loss of definition some breaking up.

Name/time	Grain	Epidermis	Lower ORS	Upper ORS	Bulb
Purafect 4 hours	blue (darker at grain surface).	Purple some sloughing.	Light purple heavy loss of definition.	Purple.	Purple heavy loss of definition.
Purafect 8 hours	blue (darker at grain surface).	Purple some sloughing.	Mostly broken up some staining light purple.	Purple.	Very light purple heavy loss of definition.
Purafect 16 hours	Blue.	Only traces of purple stained material remains.	Mostly broken up some staining light purple.	Some purple fragments remain.	Very light purple heavy loss of definition.
Purafect 20 hours	Mostly unstained except surface	Dark blue fragments remain.	Mostly broken up some staining light purple.	Some purple fragments remain.	Very light purple heavy loss of definition.
Protease 200 4 hours	Blue (darker at grain surface).	Dark purple.	Light purple.	Dark purple consistent with epidermis.	Dark purple to black.
Protease 200 8 hours	Blue (darker at grain surface.	Dark purple.	Light purple.	Dark purple consistent with epidermis.	Dark purple.
Protease 200 16 hours	Blue (darker at grain surface).	Purple some sloughing.	Light purple heavy loss of definition.	Dark purple sloughing consistent with epidermis.	Very light purple heavy loss of definition.
Protease 200 20 hours	Blue (darker at grain surface).	Purple heavy sloughing.	Light purple complete loss of definition.	Dark purple sloughing consistent with epidermis.	Light purple complete loss of definition.
Promod 4 hours	Blue (darker at grain surface).	Dark purple.	Light purple.	Dark purple consistent with epidermis.	Dark purple to black.
Promod 8 hours	blue (darker at grain surface).	Nuclei stained; only some breaking up.	Light purple some breaking up.	Dark purple consistent with epidermis.	Purple some loss of definition.
Promod 16 hours	blue (darker at grain surface).	Only traces of purple stained material remains.	Mostly broken up some staining light purple.	Some purple fragments remain.	Very light purple heavy loss of definition.
Promod 20 hours	Light blue heavy damage.	Only traces of purple stained material remains.	Light purple heavy loss of definition. Mostly broken up.	Light purple.	Very light purple heavy loss of definition.

5.3.3 Glycosaminoglycans changes - Summary

The changes in colour displayed in the fixed samples over time during the depilation process indicated there was a gradual removal of glycoproteins during depilation. Of particular interest is the staining of the lower ORS and wool bulb. Both these regions contained high concentrations of glycoproteins at the beginning of the process but as depilation proceeded the glycoproteins were lost, and the structure became less defined. Although a protease would not be expected to have activity against glycosaminoglycans activity so it is likely that the proteolytic destruction of glycoproteins such as fibronectin and or laminin may be responsible for the changes in glycan staining observed. If glycoproteins were destroyed then it is likely that the associated glycosaminoglycans would be able to be washed out and hence the staining would be different. Previous work has shown that the removal proteoglycans is important for the final leather product (Alexander *et al.*, 1986) and it has been suggested that proteoglycans are important in the unhairing process (Cantera, 2001c). This work confirms that proteolytic depilation does involve changes in the glycosaminoglycans complement within skin during depilation and is probably associated with the destruction of glycoproteins as previously found (Cantera *et al.*, 2003). Since the changes in glycosaminoglycans and depilation were achieved though the use of proteolytic enzymes it is likely that although additional glycosidic activity may be beneficial to the overall rate of depilatory activity as suggested by previous authors (Jian *et al.*, 2007), (Thanikaivelan *et al.*, 2006), (Aravindhan *et al.*, 2004), (Cantera, 2001c), (Cantera *et al.*, 2003), (Sivasubramanian *et al.*, 2008) it may not be necessary for a successful non-damaging enzyme depilatory.

5.4 Individual protein changes during depilation

A major purpose of beam-house processing is the removal of non-collagenous proteins from skin (Sharphouse, 1971),(Kronick & Iandola, 1998). The removal of epidermis is a requirement of beam-house processing to “clean up” the grain surface, because residual epidermis (or scud) that persists to the crust stage is unsightly and down-grades the value of the leather.

When correctly carried out, bulk destruction of the epidermis by lime sulfide processing is generally successful in removing the scud. It has previously been assumed that all non-collagenous proteins in skin, including those involved in attaching the epidermal cells and cornified layer to the dermis, are destroyed along with the other keratinaceous material during conventional liming. It has been shown, however, that some minor proteins are not removed during this process ((Kronick & Iandola, 1997); (Kronick & Iandola, 1998)). The implication is that some minor proteins, which are involved in holding the scud to the dermis, may remain attached to the grain enamel throughout processing, and may be an integral part of the grain enamel. Alternatively, they may be required to be removed to ensure a clean grain.

Only some of the minor proteins have been monitored throughout the beam-house process ((Allsop *et al.*, 2005), (Kronick & Iandola, 1998)). It is not clear what role the minor proteins that remain in the skin through to pickle play in the final leather.

Fibronectin is a minor skin protein which is associated with cell attachment and is located within the basement membrane (Rollins *et al.*, 1982). It exists as fibrils and aggregates in native skin (Rollins *et al.*, 1982). Five percent (5%) of its molecular weight is due to covalently linked carbohydrate chains that may protect the protein during processing (Hynes, 1999).

Laminin, which is also a minor protein of skin, is located in the basement membrane and is associated with interactions between the cell and the extracellular matrix (Sasaki & Timpl, 1999). It has also been shown to be associated with separation of epidermis from dermis *in vivo* (Saksela *et al.*, 1981). Laminin has also been detected at the edge of the dermal side of tryptase induced blisters ((Kaminska *et al.*, 1999)) indicating involvement in lesion formation. It may therefore play a role in skin processing and be a component of the grain enamel after beamhouse processing.

In this section, immunohistological methods were employed to identify which minor skin proteins were present in good quality skins, but missing in damaged skins.

5.4.1 Immunohistology methods and materials

Immunohistology has previously been used to investigate connective tissue components in bovine hides and kangaroo skins (Stephens *et al.*, 1991) and more recently in deer and pig (Fukunaga *et al.*, 2006). In the present work, samples of ovine skin were examined using immuno-histology in order to investigate the location and orientation of a number of proteins during beam-house processing.

The literature review pointed to a number of proteins that might be expected to remain in the skin during and after processing. These included: laminin, fibronectin, collagens III, IV, V, VI, and VII, and elastin. Antibodies to these proteins were therefore obtained in an attempt to investigate the position, importance and persistence of these proteins in both conventional and enzymatic depilation.

A list of the protein antibodies that were made available within this work is given in Table 21. A selection of techniques for preparing samples for immuno-histology were trialled in an attempt to generate positive staining results for each of the antibodies tested.

Table 21: Antibodies available for examination of the depilation process

Enzyme	Raised in	Clone	Supplier Product	Lot	Dilution
Fibronectin	Mouse	IST3	Sigma F0791	033K4882	1:200
Laminin	Mouse	LAM89	Sigma L8271	073K4879	1:200
Elastin	Mouse	BA-4	Sigma E-4013	094K4773	1:200
Collagen III	Mouse	FH-7A	Sigma C-7805	12K4861	1:50 to 1:10000
Collagen IV	Mouse	COL94	Sigma C1926	054K4806	1:50 to 1:10000
Collagen VI	Rabbit		Chemicon AB7822		1:200
Collagen VII	Mouse	LH7.2	Sigma C6805	094K4801	1:200

The only antibodies which were sheep specific were fibronectin, elastin and collagen type VII. The other antibodies were tested in anticipation that they may have some species cross reactivity.

Prepared sections were either immediately subjected to the immunohistology protocol or an epitope retrieval procedure involving either: citrate buffer (section 8.3.7.1) or trypsin (section 8.3.7.2). These methods were chosen because they provided a simple protocol for successful staining of a large portion of the immunohistology samples.

Trypsin epitope retrieval combined with the use of paraffin embedded formalin-fixed sections provided the most successful method for positive staining. Unfortunately no positive staining was observed with either the collagen III or collagen IV antibodies using any combination of the described techniques and a wide range of dilutions of the primary antibody (1:50-1:10000). It is likely that those antibodies had no cross reactivity to sheep and so those antigen proteins were not investigated in this section. Trypsin epitope retrieval from paraffin embedded formalin-fixed sections produced positive staining for all the other proteins using a primary antibody dilution of 1:200. Samples of skin undergoing conventional depilation and enzymatic depilation were examined and the results for each are described in the following sections.

Conventionally processed skin samples were prepared from lambskins at different stages of beam-house processing including green skin, painted and pulled skin, and pickled pelt. For enzymatically depilated samples lambskins were prepared for depilation using Protease 6L and Purafect 4000L as described in section 3.2.1. Skin samples were taken at 4, 8, 16, and 20 hours after exposure to the enzyme, and after depilation and pickling using the standard pickle as described in section 8.2.3.

A biotin-streptavidin detection system was used as described in section 8.3.6. Briefly: Sections were cut from samples fixed in buffered formalin (0.0375 M phosphate buffer at pH 6.65 in 0.8% w/w formaldehyde) for 24 h before being processed through to paraffin (section 8.3.1.4) and embedded in paraffin wax. Five micron sections cut from the prepared samples, were then floated on warm water to enable their attachment to glass slides which were then air-dried overnight for immuno-histo-chemical (IHC) analysis. The biotin-streptavidin detection system was used as follows: Sections were de-paraffinised by immersing in two changes of xylene for 6 minutes each followed by absolute alcohol for 1 minute, then 95% v/v alcohol for 1 minute then 70% v/v alcohol for 1 minute, then tap water for 1 minute. The sections were then equilibrated in

phosphate buffered saline (PBS) (0.01M Phosphate NaCl 9.0 g/L), pH 7.2 for 1 min. The effects of endogenous peroxidase were removed by exposure of the sections to 3% hydrogen peroxide in PBS for 30 min after which they were washed and re-equilibrated in PBS for 1 min.

An area on the slide containing the section was encircled with a PAP pen (Sigma) to create a fluid barrier to hold the reagents. Non-specific binding sites were blocked by covering the section with 1% bovine serum albumin (BSA Sigma) and allowing the slide to stand for 5 min, before incubating it in conditions of high humidity at room temperature for 1 h. Conditions of saturated humidity were obtained by placing the slide in a sealed chamber containing a paper towel wetted with tap water. The sections were then drained and washed for 1 min in each of three changes of PBS.

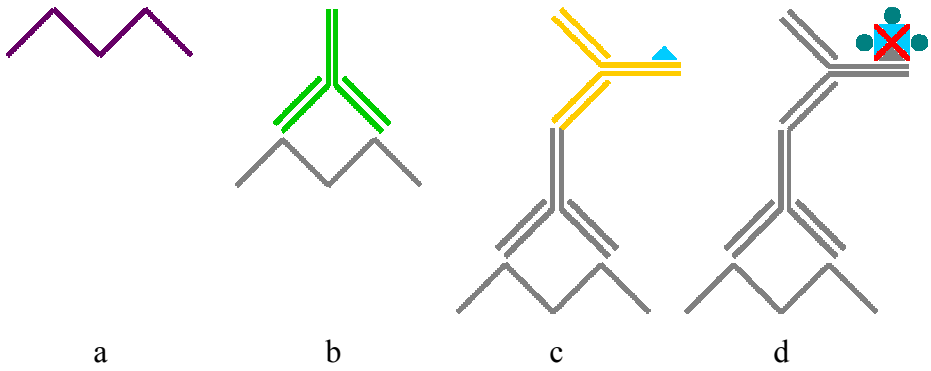


Figure 62: Immunohistological conceptual diagram modified from (Boenisch, 2001), a) antigen, b) primary antibody attached, c) biotinylated secondary antibody attached, d) preformed streptavidin biotinylated horseradish peroxidase complex attached.

Antibodies (Table 21 diluted in PBS) were then added to the sections (20 uL) and again incubated at high humidity for 1 h after which they were drained and washed with three separate changes of PBS. Approximately 100 uL biotinylated secondary antibody (either Amersham anti-rabbit from sheep (RPN1004) or anti-mouse from sheep (RPN1001) diluted 1:200 in BSA) was added and the sections were incubated at high humidity for 30 min. They were then drained and washed in three changes of PBS before being exposed to a preformed biotin-streptavidin-peroxidase complex (Streptavidin biotinylated horseradish peroxidase RPN1051 diluted 1:200 in BSA). A conceptual diagram of the immunohistological procedure is given in Figure 62.

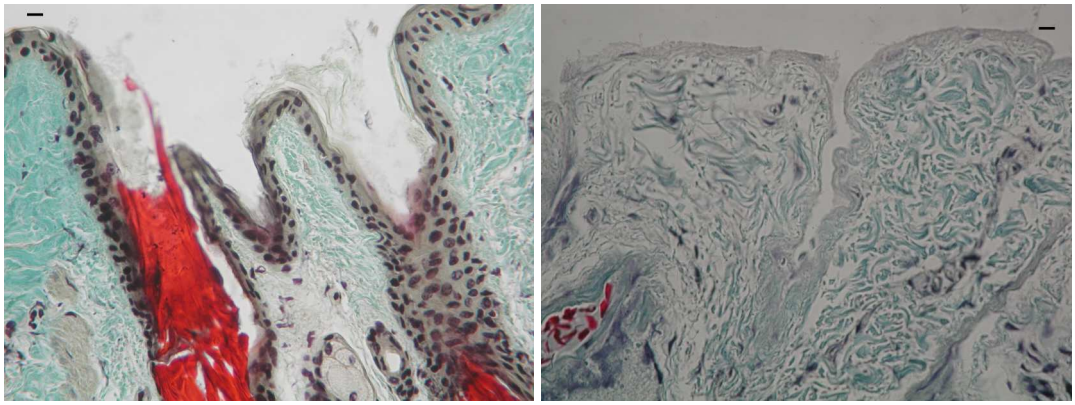
Sections were then drained and washed in three changes of PBS before flooding with 10 mL of 0.5 mg/mL 3,3 diaminobenzidine (DAB), containing 20 uL of 30% peroxide for approximately 3 min. It was found at this point that it was particularly important to use fresh hydrogen peroxide and to make the DAB solution up fresh immediately before application as standing for even a few minutes would impair the peroxidase reaction. The colour was allowed to develop for approximately 3 min before the reaction was halted by immersion of the sections in PBS. The sections were rinsed in tap water before being counter stained with Mayer's hämalaun for 1 min, after which they were rinsed in tap water, dehydrated, cleared and mounted in DPX mounting medium. The sections were examined under a light microscope at 40x magnification where the primary protein of interest was visualised as a brown/black stain.

The dilution of the primary antibody was determined by carrying out a standardisation of the above procedure on serial sections of raw skin and examining the quality of staining at primary antibody dilutions of 1:1000, 1:500, 1:200, and 1:100. For each of the proteins that provided a positive reaction, the best dilution was 1:200.

5.4.2 SACPIC controls

In order to properly assess the relationship between the minor proteins that were identified and the process of enzyme depilation a series of SACPIC stains were again carried out, this time on the thin sections that were prepared through the paraffin process for immuno-histology. Sections prepared for immunohistochemistry using the paraffin embedding technique were stained with the SACPIC stain as described in section 8.3.3 to directly compare with detection of specific proteins using immunohistochemistry.

The location and condition of structures at the grain surface in green skin can be seen in the photomicrograph illustrated in Figure 63a and contrasted with a typical sample of enzyme depilated material (Figure 63b). As the structures changed during the depilation process, the condition of the structures was recorded and is displayed in Table 22.



a) b)

Figure 63: SACPIC stain of thin cross sections (bar is 10 um). Collagen stained green, cell nuclei stained black, and the inner root sheath is stained red. a) raw skin clearly showing the inner root sheath in red and epidermal cells in black, b) Skin exposed to Purafect for 8 hours showing removal of epidermal cells and most root sheath cells.

Even on the thin sections used in this section of work the results did not show any definitive region of breakage between the epidermis and the enamel surface. The epidermis was either intact or sloughed off. The description of the structural changes occurring in the skin during enzyme depilation as observed on sections cut to 5 um thickness is the same as those observed on thicker sections prepared in section 5.2.3.

However on the thinner sections it was easier to visualise the inner root sheath which was cut through in contrast to the thicker sections in which a number of whole wool roots were visible through the depth of the section. The immunohistochemistry results were compared directly to the SACPIC stained thin section controls that have been described in this section

Table 22: Enzymatically depilated skins; SACPIC stained structural controls. Collagen stained green, cell nuclei stained black, and the inner root sheath is stained red.

Sample /time	Epidermis	Enamel	Grain	Root Sheath	Wool bulb	Corium
Green skin	Cornified material visible above epidermal surface. Epidermis intact. Grey with black cells.	Intact but hard to distinguish below epidermis. Forest green.	Intact smooth ordered structure. Forest green.	ORS intact s and stained the same as the epidermis. Inner root sheath intact and stained red.	Amorphous, dark blue stain with some black spots.	Intact smooth ordered structure. Stained blue green.
Protease 6L 4 hours	Some cornified material still visible above epidermal surface. Epidermis mostly intact.	Intact but hard to distinguish below epidermis.	Some regions altered in shaped appearing collapsed.	Some minor breakdown in IRS. ORS mostly intact.	Some amorphous material gone, black spots remain.	Intact smooth ordered structure. Stained blue green.
Protease 6L 8 hours	No cornified material remains most epidermis gone.	Some isolated fraying observed. Enamel region no longer stained green.	Some regions altered in shaped appearing collapsed (a greater area than observed at 4 hours).	Only isolated red fragments remain. Cells of ORS mostly gone and structure generally collapsed.	Some amorphous material gone, black spots remain. Thin fibres remain in place of amorphous material.	Some splitting up of collagen fibres. Frayed appearance on some fibres.
Protease 6L 16 hours	All epidermis gone.	Large regions of fraying. Some enamel pealing off from the grain.	Most of the grain appears collapsed having lost its smooth native structure.	Completely destroyed only visible as distinctive region of collagen fibre layout in grain.	Most amorphous material gone, black spots remain, now angular in appearance. Thin fibres remain in place of amorphous material.	Splitting up of collagen fibres. Frayed appearance on some fibres.
Protease 6L 20 hours	All epidermis gone.	Large regions of fraying. Some enamel pealing off from the grain.	Most of the grain appears collapsed having lost its smooth native structure.	Completely destroyed only visible as distinctive region of collagen fibre layout in grain.	Most amorphous material gone, black spots remain, now angular in appearance. Thin fibres remain in place of amorphous material.	Splitting up of collagen fibres. Frayed appearance on some fibres.
Protease 6L pickled pelt	All epidermis gone.	Large regions of fraying. Some enamel pealing off from the grain.	Most of the grain appears collapsed having lost its smooth native structure.	Completely destroyed only visible as distinctive region of collagen fibre layout in grain.	Most amorphous material gone, black spots remain, now angular in appearance. Thin fibres remain in place of amorphous material.	Splitting up of collagen fibres. Frayed appearance on some fibres.

Sample /time	Epidermis	Enamel	Grain	Root Sheath	Wool bulb	Corium
Purafect 4000L 4 hours	Some cornified material still visible above epidermal surface. Epidermis mostly intact.	Intact but hard to distinguish below epidermis.	Some regions altered in shaped appearing collapsed.	Some minor breakdown in IRS. ORS mostly intact.	Some amorphous material gone, black spots remain.	Intact smooth ordered structure. Stained blue green.
Purafect 4000L 8 hours	No cornified material remains most epidermis gone.	Some isolated fraying observed. Enamel region no longer stained green.	Some regions altered in shaped appearing collapsed (a greater area than observed at 4 hours).	Isolated red fragments remain mostly gone. Cells of ORS mostly destroyed and structure generally collapsed.	Some amorphous material gone, black spots remain. Thin fibres remain in place of amorphous material.	Some splitting up of collagen fibres. Frayed appearance on some fibres.
Purafect 4000L 16 hours	All epidermis gone.	Large regions of fraying. Some enamel pealing off from the grain.	Most of the grain appears collapsed having lost its smooth native structure.	Completely destroyed only visible as distinctive region of collagen fibre layout in grain.	Most amorphous material gone, black spots remain, now angular in appearance. Thin fibres remain in place of amorphous material.	Splitting up of collagen fibres. Frayed appearance on some fibres.
Purafect 4000L 20 hours	All epidermis gone.	Large regions of fraying. Some enamel pealing off from the grain.	Most of the grain appears collapsed having lost its smooth native structure.	Completely destroyed only visible as distinctive region of collagen fibre layout in grain.	Most amorphous material gone, black spots remain, now angular in appearance. Thin fibres remain in place of amorphous material.	Splitting up of collagen fibres. Frayed appearance on some fibres.
Purafect 4000L pickled pelt	All epidermis gone.	Large regions of fraying. Some enamel pealing off from the grain.	Most of the grain appears collapsed having lost its smooth native structure.	Completely destroyed only visible as distinctive region of collagen fibre layout in grain.	Most amorphous material gone, black spots remain, now angular in appearance. Thin fibres remain in place of amorphous material.	Splitting up of collagen fibres. Frayed appearance on some fibres.

5.4.3 Location of skin minor proteins and their presence in conventionally processed material results

The location of all of the minor proteins identified using immunohistology in this work were assessed for their persistence during conventional processing in order to determine their likely importance in enzyme depilation. This cross sections were observed and are the basis for the illustrations shown. The results are given in the following sections.

5.4.3.1 Location of laminin and its response to conventional processing

The review of the literature indicated that laminin may be related to cell adhesion and its destruction may be associated with an enzymatic depilation process. Its presence in ovine skin and during the depilation process has not been previously investigated and so it was important to locate laminin in ovine skin relative to the enzyme depilation process. Unfortunately the staining was relatively poor for laminin and the positive staining observed on the original sections is even less obvious in the photomicrograph reproductions, so its location in the skin strata could not be unequivocally defined.

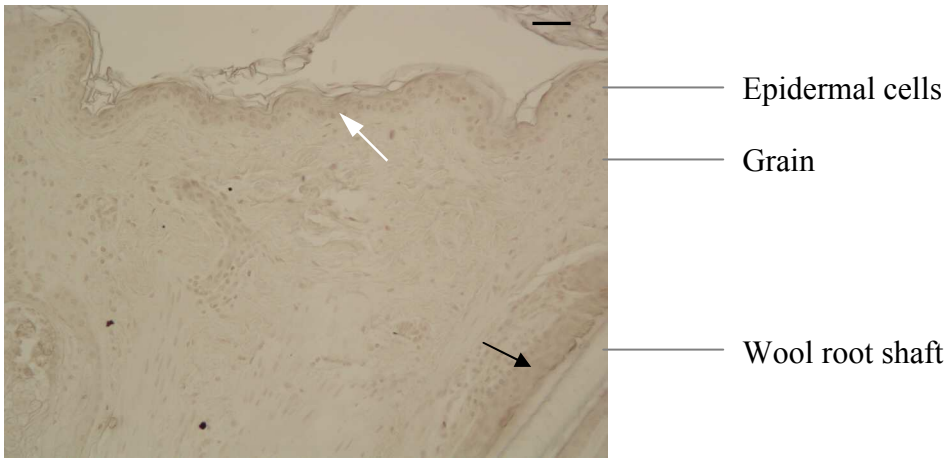


Figure 64: Immunohistology of laminin in raw skin. Epidermal cells are visible at the top (white arrow) and a wool root sheath visible on the bottom right, note the positive staining occurring around the cells of the inner root sheath (black arrow) (bar is 50 um).

It was noted that significant staining occurred around the cells of the inner root sheath as illustrated in Figure 64. While the photomicrograph does not have the contrast of the original section the positive staining observed around the wool root sheath (black arrow Figure 64) was different to the other regions of staining when viewed on the original section.

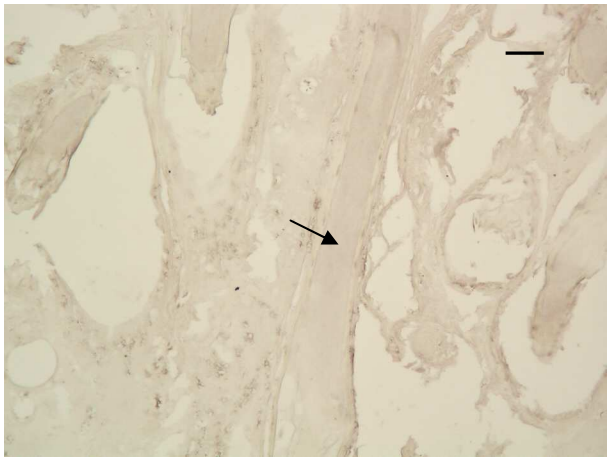


Figure 65: Immunohistology of laminin in a conventionally depilated slat with a wool root visible as the vertical structure in the image centre, note again the presence of positive staining around cells of the inner root sheath (arrowed)(bar is 50 um).

Laminin can still be seen in the conventionally depilated material (Figure 65) located around the cells of the inner root sheath. Again the positive staining of laminin is more easily observed on the original sections around the wool root than in the photomicrographs as presented.

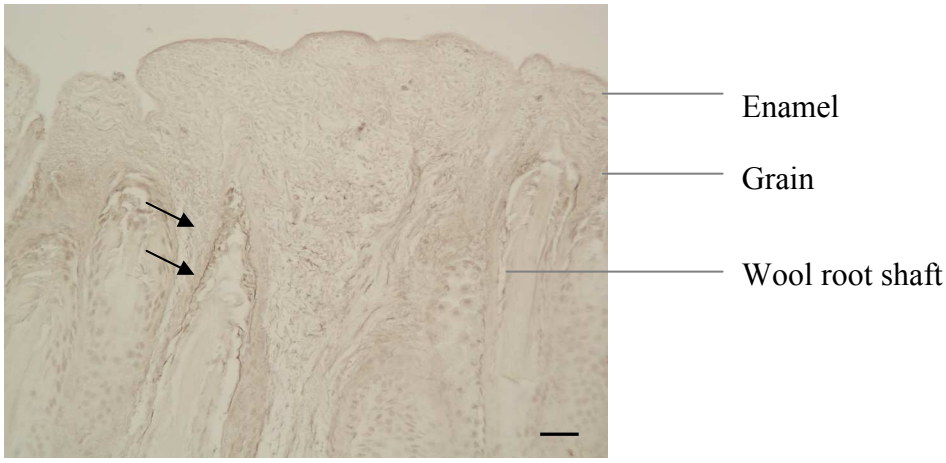


Figure 66: Immunohistology of laminin in a conventionally processed skin after deliming and bating. Note the positive staining in the cells of the root sheath (arrowed) (bar is 50 um)

The deliming and bating process resulted in samples that when sectioned gave a greater level of response to the immunohistological staining procedure, possibly through the exposure of antigenic sites by the action of the proteolytic bate. Positive staining can be viewed around the cells of the root sheath indicating the presence of laminin in this area.

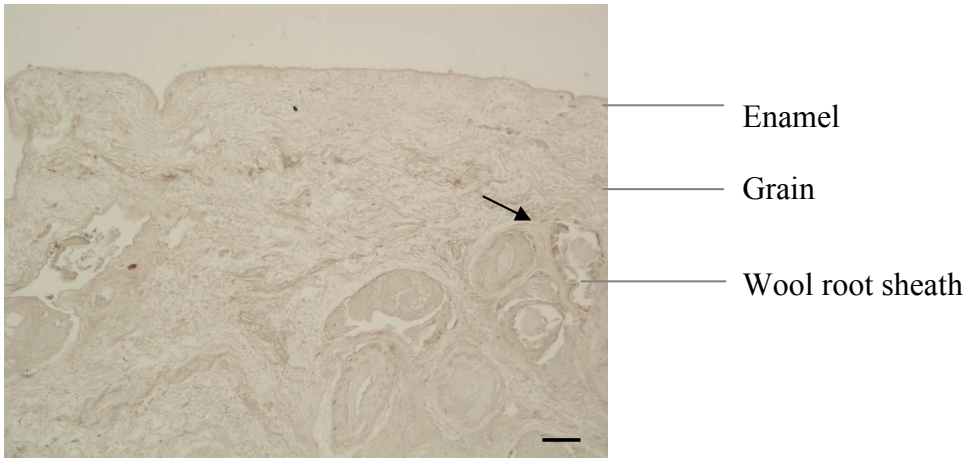


Figure 67: Immunohistology of laminin in a conventionally processed skin after pickling. Note the positive staining in cells of the root sheath (arrowed) (bar is 50 um)

Immunohistological methods showed that laminin was not present within the grain surface. While somewhat difficult to see in the photomicrographs, positive staining was observed around the cells of the root sheath, which persisted through to the pickle stage. This indicated that, while probably not important for the surface characteristics of the final product, the destruction of laminin may assist the enzymatic depilation process.

5.4.3.2 Location of collagen VI and its response to conventional processing

In the literature review it was noted that collagen VI may play a role in the structure of collagen I fibrils particularly the layout of the fibril structure. It was also noted that collagen VI persisted through conventional processing. It was important, therefore, to locate this protein within the skin so that its relevance to enzymatic processing could be evaluated. Results for immunohistological staining for collagen VI are illustrated in the following figures.

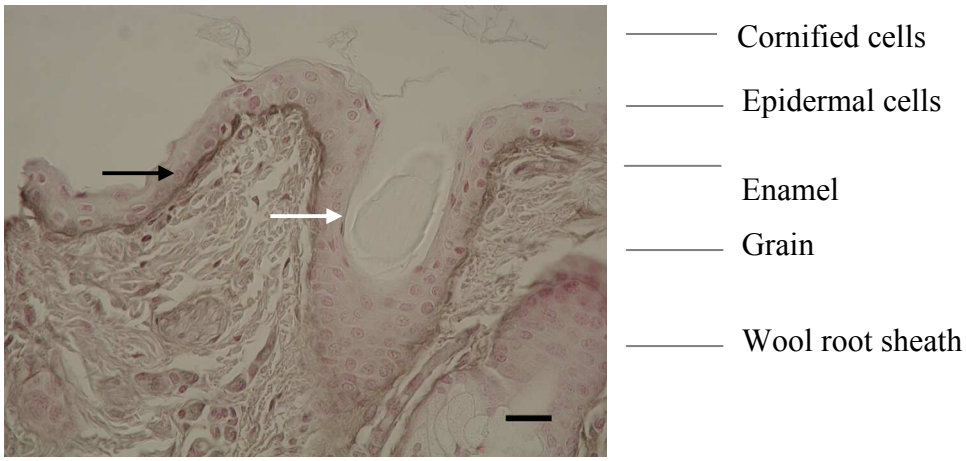


Figure 68: Immunohistology of collagen VI in raw skin with epidermis visible as the pink cells at the top of the image and a wool fibre visible in the centre right of the image (white arrow). Note the positive staining in the enamel region below the epidermal cells (black arrow) (bar is 25 um)

When a sample of raw skin was examined (Figure 68) positive staining was evident in the enamel when sections were probed using antibodies for collagen VI. Cornified cells suggests that this particular collagen may be important in maintaining the fine collagen structure found in the enamel, and therefore be important to the appearance of the final product.

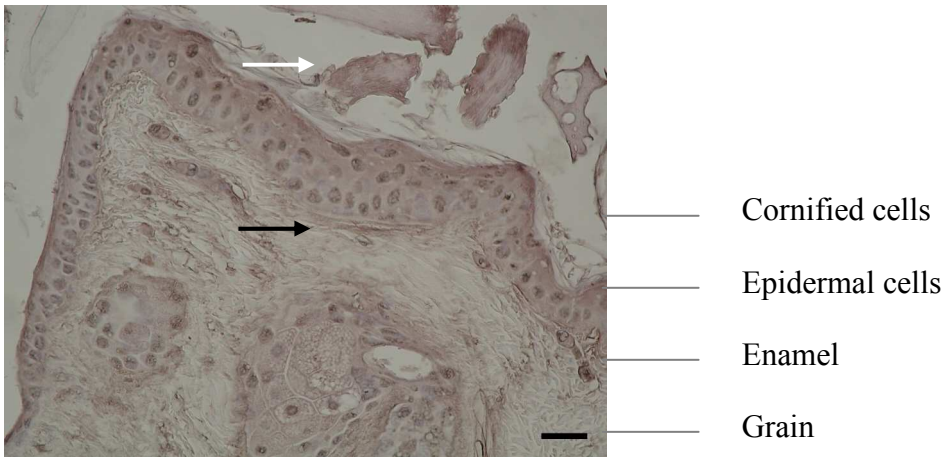


Figure 69: Immunohistology of collagen VI in a conventionally depilated slat prior to deliming and bating. Pieces of the epidermal layer have become free and can be seen at the top of the image along with cornified cells (white arrow). Note the positive staining in the enamel region (black arrow) (bar is 25 um)

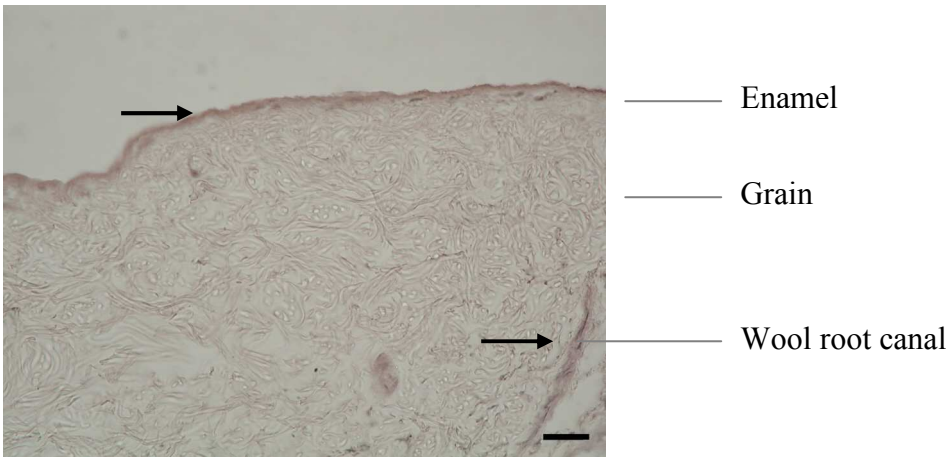


Figure 70: Immunohistology of collagen VI in a conventionally processed skin after deliming and bating. Note the positive staining in the enamel region and down the side of a wool root canal (black arrows). While the intensity of staining is less clear there is clearly positive staining within the enamel region. (bar is 25 um)

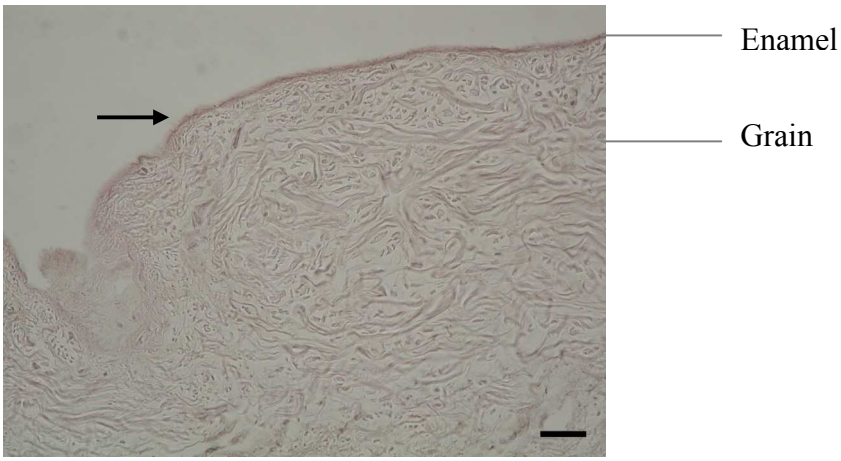


Figure 71: Immunohistology of collagen VI in a conventionally processed skin after pickling. Note the positive staining in the enamel region (arrowed). While the intensity of positive staining has decreased compared to the delimed and bated skin there is positive staining within the enamel region. (bar is 25 um)

Overall it is evident that collagen VI occurs within the grain enamel and persists through processing, to the pickle stage in a conventionally processed skin.

5.4.3.3 Location of collagen VII and its response to conventional processing

It was noted in the literature review that collagen VII plays a major role in the adhesion of extracellular matrix components. It was therefore important to locate it within ovine skins during conventional processing so that it could be determined whether is was

important for the enzymatic depilation process. Collagen VII did not appear to be located in the regions of interest in terms of enzymatic depilation Figure 72.

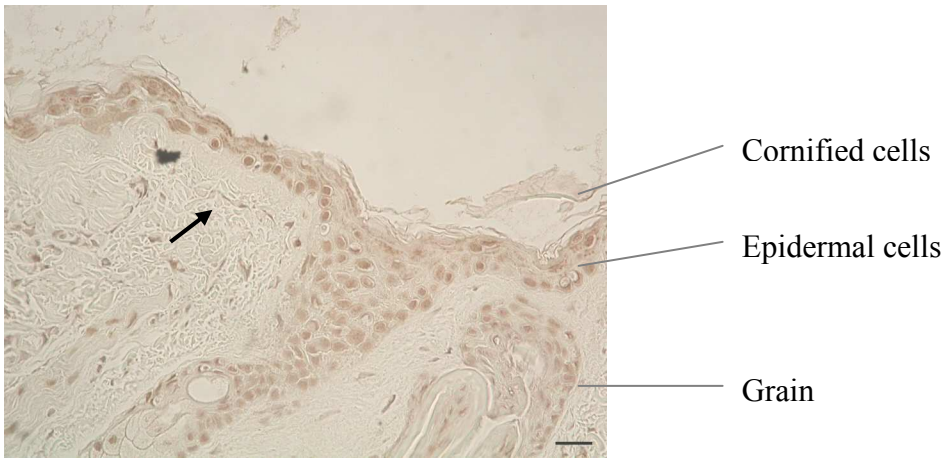


Figure 72: Immunohistology of collagen VII in raw skin. Note some positive staining between some epidermal cells and the small plaques in the grain (arrowed) not to be mistaken for the cells that are counter stained (bar is 25 um).

It was not evident around the cells of the root sheath nor was it found within the grain surface indicating that it is probably not associated with the grain surface damage associated enzymatic depilation. Small plaque like regions of positive staining were observed throughout the grain structure. Again the positive staining is difficult to differentiate from the counterstaining visible in the photomicrographs but could be seen in the original sections.

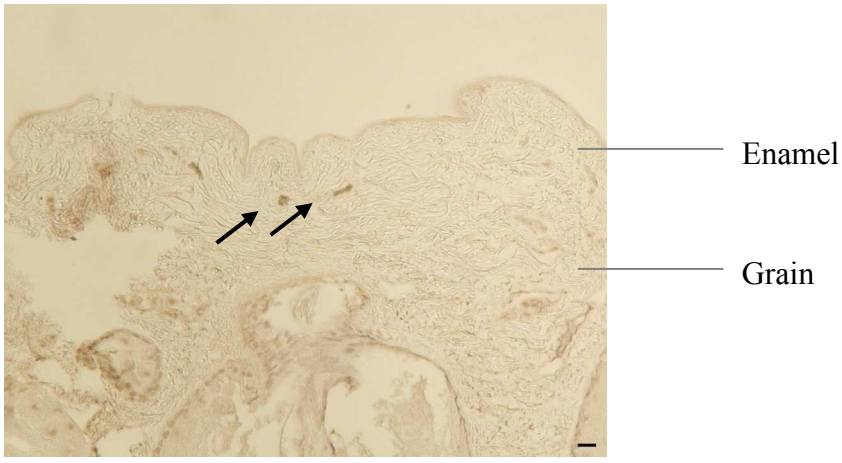


Figure 73: Immunohistology of collagen VII in conventionally depilated material. Again positively stained plaques differentiable in the original sections were located in the grain (arrowed). Unfortunately these are not differentiable from the counterstained cells in the photomicrograph (bar is 20 um)

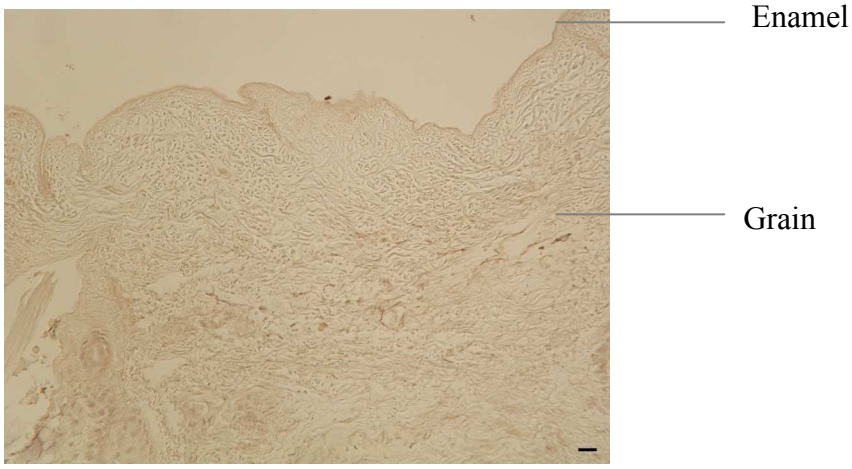


Figure 74: Immunohistology of collagen VII in a conventionally processed skin after deliming and bating. The positively stained plaques visible earlier in processing cannot be seen in the delimed and bated material (bar is 20 um)

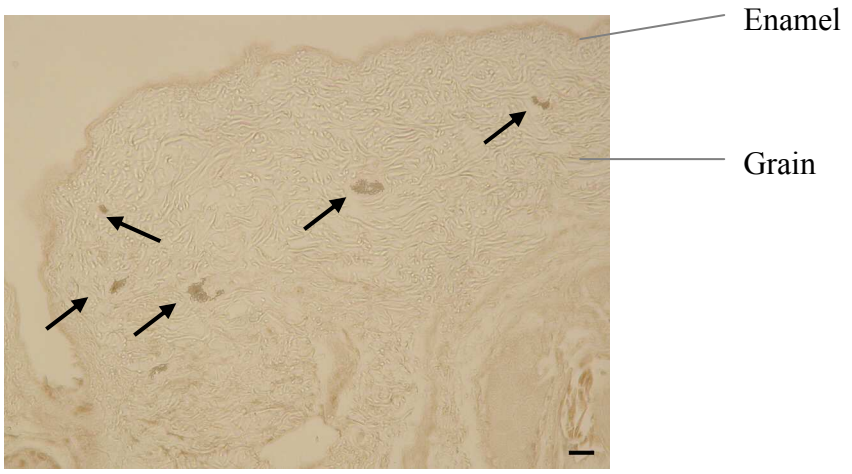


Figure 75: Immunohistology of collagen VII in a conventionally processed skin after pickling. Note the positively stained plaques in the grain (arrowed) (bar is 20 um).

Plaques of material that stained positivelyusing antibodies against collagen VII were found to persist throughout enzyme depilation through to the pickled pelt product. However since staining was not evident within regions of interest for enzymatic depilation the protein is unlikely to play an important role in either the depilation process or in the damage observed in the enamel layer. It might be interesting for future

research to investigate the effect of the persistence of this protein on the quality of the final leather but that lies outside the scope of this work.

5.4.3.4 Location of fibronectin and response to conventional processing

It was noted in the literature review that fibronectin also plays a role in cell adhesion in the extracellular matrix. Its location and persistence in conventional processing has not previously been described so it was important to locate it in ovine skin during conventional processing so that its relevance to enzymatic depilation could be ascertained. Fibronectin was observed as a fine network around fibroblasts and collagen fibre bundles. It appeared to be distributed throughout the grain and corium and although no particular location was favoured, there appeared more present in the grain than in the corium, and it appeared to be concentrated around non-fibrillar regions.

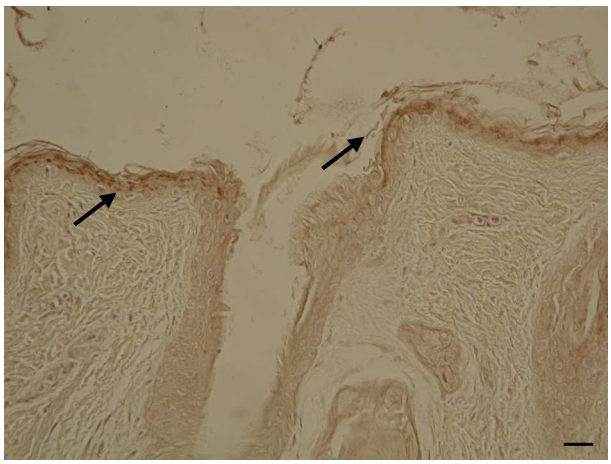


Figure 76: Immunohistology of fibronectin in raw skin. A wool root shaft is clearly visible in the centre of the image with epidermal cells at the top and wool root sheath cells descending into the wool root canal. Note the positive staining located amongst the cells of the epidermis (arrowed). (bar is 20 um).

In addition, positive staining was particularly prevalent amongst the cells of the epidermis as shown in Figure 76. There was less staining, however, between the cells of the root sheath.

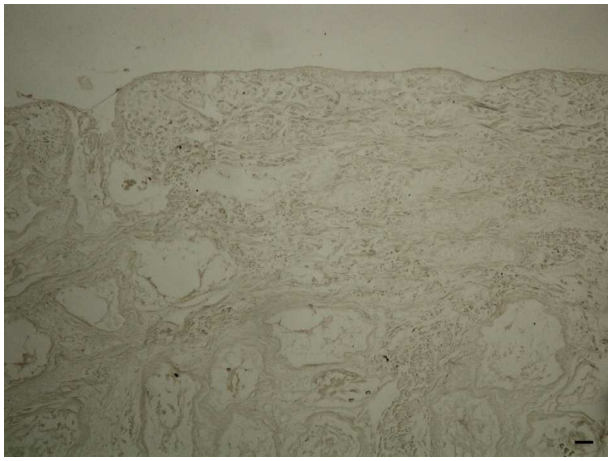


Figure 77: Immunohistology of fibronectin in conventionally depilated skin. Note the lack of positive staining now that the epidermis is gone. Note that the microscope light intensity was adjusted in an attempt to maximise any possible staining. (bar is 20 um).

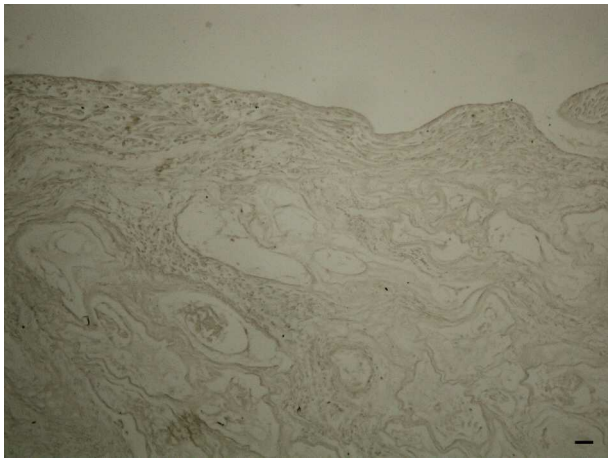


Figure 78: Immunohistology of fibronectin in conventionally delimed and bated skin. Note the lack of positive staining now that the epidermis is gone. Note that the microscope light intensity was adjusted in an attempt to maximise any possible staining. (bar is 20 um).

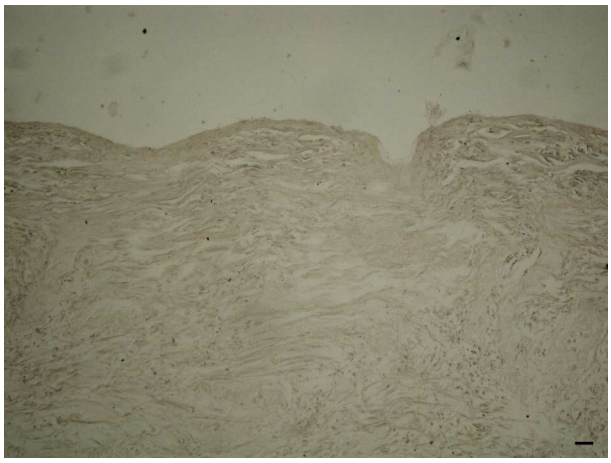


Figure 79: Immunohistology of fibronectin in conventionally processed pickled pelt. Note the lack of positive staining now that the epidermis is gone. Note that the microscope light intensity was adjusted in an attempt to maximise any possible staining. (bar is 20 um).

Overall fibronectin appears to be a particularly important protein because it appears to be located in the regions that are destroyed during the enzyme depilation process. Furthermore, it did not appear to be located in the regions of the grain enamel that were damaged after completion of the depilation process. It would seem from the results, that fibronectin might be an important target for enzymatic depilation. Destruction of the fibronectin network might loosen the wool fibre without damaging other proteins. Staining, however, was less pronounced in the region of the wool root sheath indicating that an enzyme that targeted this protein alone may have difficulty in destroying the ”cement” of the wool root sheath and result in incomplete depilation even though complete removal of the epidermis may occur.

5.4.3.5 Location of elastin and response to conventional processing

As stated in the literature review elastin is an important protein in the conversion of skin into leather. The method of viewing elastin using immuno-histology that was used for other minor proteins was also used for elastin to clarify its location in skin during conventional processing. Positive staining indicated elastin throughout the grain which appeared as horizontal fibres a few microns in diameter. In the grain enamel the elastin appeared to take the form of vertical fibres with a smaller diameter branching out and up to the epidermal dermal junction.

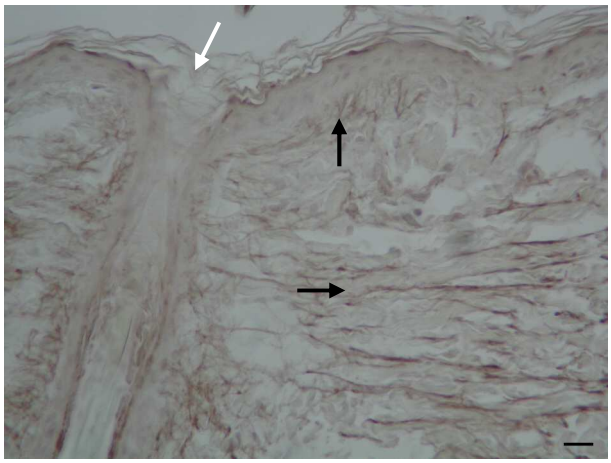


Figure 80: Immunohistology of elastin in raw skin, containing a wool root (white arrow). Note the horizontal fibres in the grain and vertical fibres in the enamel (black arrows) (bar is 100 um)

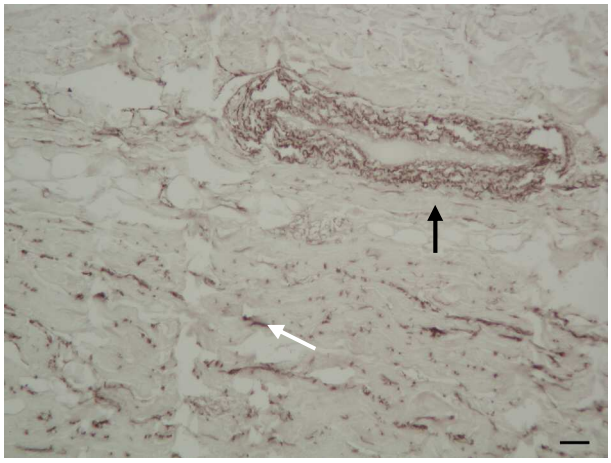


Figure 81: Immunohistology of elastin in depilated skin. Note the fibres around a collapsed blood vessel (black arrow), and the broken elastin fibres deeper in the grain (white arrow). (bar is 100 um)

Elastin fibres were more difficult to visualise in the grain surface after conventional depilation but were still visible mostly as broken fragments deeper in the grain and around blood vessels as shown in Figure 81.

After deliming the elastin was seen in the grain as horizontal fibres with some broken pieces (Figure 82). Again the elastin visible in the enamel existed as vertical fibres. It is worth noting that the depth of sample containing these vertical fibres appears to be

about 100 um, which is about the thickness of the grain enamel as noted in the literature review section 2.5.

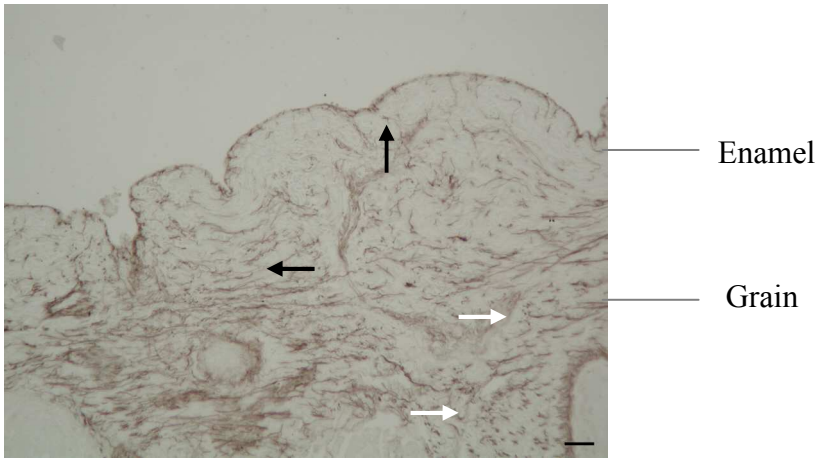


Figure 82: Immunohistology of elastin in delimed skin. Note the horizontal fibres in the grain and vertical fibres in the enamel (black arrows). Note also the presence of broken fibres (white arrows). (bar is 100 um)

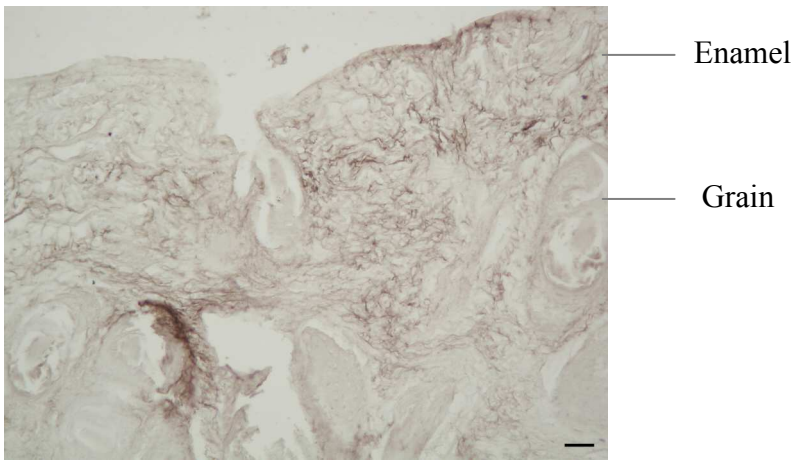


Figure 83: Immunohistology of elastin in pickled pelt. Note the lack of staining in some regions of the surface and the broken material scattered throughout. (bar is 100 um)

Elastin was still visible in the pickled pelt as some scattered broken horizontal fibres in the grain and some vertical fibres in the enamel. However, some regions did not show any staining indicating the absence of elastin in these regions. This follows earlier work described in section 2.6.5.1 where elastin appeared to be removed from the upper layer of the grain during processing depending on the type of bate used. When a pancreatic (or trypsin based) bate was used, as in the "standard" process applied here (section 8.1), the elastin stays relatively intact, whereas when alternative bates were used more elastin

appeared to be destroyed resulting in poorer quality leather (Lowe, 1997); (Allsop *et al.*, 2005).

Since the removal of elastin is related to faults that occur during processing (Allsop *et al.*, 2005) it would be interesting to investigate the changes in elastin structure that occur during enzyme depilation. However, it would seem that, while the removal of elastin has been noted as a possible cause of faults that develop during processing, the nature of its presence in the pelt and its partial removal from the grain surface during conventional processing indicate that it is unlikely to be associated directly with the dewooling process and is also unlikely to be related directly to the surface damage.

5.4.3.6 Conclusions for the location of minor proteins

Fibronectin and laminin are fibrillar proteins of the extracellular matrix that were observed to be associated with the cells of the epidermis and the wool root sheaths. Targeting these extracellular proteins with specific enzymes may therefore lead to a successful depilation.

Collagen VI, collagen VII, and elastin all appeared to persist right through the conventional process to the pickled pelt stage. It was also found that regions of the grain contained collagen VI indicating that this molecule might play an important role in the integrity of the grain enamel. Collagen VII, and elastin, on the other hand, did not appear to be associated with either regions of the skin that would be expected to be a factor in depilation or with regions that are damaged during enzyme depilation. It is already known that damage to elastin can occur during processing and that removal of too much elastin can result in a poorer quality pickled lambskin product. Therefore monitoring of this protein is still of interest during the enzymatic depilation process. It would be useful in future work to obtain antibodies against other ovine proteins of interest also including those which could not be assessed in this work such as collagen III and collagen V which may be present and associated with the fine fibres of the grain surface and collagen IV which may be associated with the destruction of the basal membrane during enzymatic depilation.

5.4.4 Minor proteins in enzyme depilated skins

Since the activities of a number of commercial enzymes against skin had already been determined, the two best performing enzymes were selected (Chapter 3). The response of the skin proteins to these enzymes was then assessed. Samples were prepared as described in section 5.4.1. with both Purafect and Protease 6L enzymes investigated and examined at 4, 8, 16, and 20 hours after exposure to the enzyme and in the pickled state. For each combination of enzyme and depilation time a large number of observations were made of the epidermis, grain enamel, grain, root sheath, root bulb, and corium. Since the total number of observations was large (330 observations; 6 locations on each section, 11 different enzyme/time samples, 5 different antigens) the results of these observations were aggregated and summarised in a table for each protein.

5.4.4.1 Changes to fibronectin during enzymatic depilation

Results from raw skin and conventional processing indicate that fibronectin surrounds the cells of the epidermis. It was of interest therefore to determine the persistence of fibronectin during enzyme depilation and to determine whether it is involved in the enzyme depilation process.

The results are summarised in Table 23. The results show that fibronectin is likely to be important in the enzyme depilation process. It was concentrated in the regions that were destroyed during the loosening stages of the depilation process. In particular it was found at the edge of the collagen matrix around the bulb, root sheath, and grain surface. When the enzyme had loosened the wool, the collagen matrix separated from the cells at these points and the fibronectin was found on the collagen matrix side. Fibronectin was also found at the outermost surface of the grain surface after enzyme depilation. These findings indicate that fibronectin would be a good target for a more specific enzyme depilation reagent. However, since fibronectin was found at the grain surface after depilation it is possible that its destruction may be related to the damage seen at the grain surface in unsuccessful enzyme depilation regimes.

Table 23: Immunohistology of fibronectin in enzymatically depilated pelts.

Sample / time	Epidermis	Enamel	Grain	Root Sheath	Wool bulb	Corium
Green skin	Small fibres around cells.	Located at epidermal edge.	Located throughout particularly associated with macro structures.	Small fibres around cells.	Surrounds bulb.	Isolated fibres.
Protease 6L 4 hours	Small fibres around cells.	Located at epidermal edge.	Located throughout.	Small fibres around cells.	Surrounds bulb.	Isolated fibres.
Purafect 4000L 4 hours	Small fibres around cells.	Located at epidermal edge.	Located throughout	Small fibres around cells.	Separation of bulb from surrounding tissue. Fibronectin appears on tissue side.	Isolated fibres.
Protease 6L 8 hours	Most epidermis gone.	Located at epidermal edge but less than previously.	Located near structures but not within destroyed regions.	Small fibres around cells. Not found around destroyed cells.	Separation of bulb from surrounding tissue. Fibronectin appears on tissue side.	Isolated fibres.
Purafect 4000L 8 hours	Most epidermis gone.	Slight staining in enamel.	Located near structures but not within destroyed regions.	Small fibres around cells. Not found around destroyed cells.	Separation of bulb from surrounding tissue. Fibronectin appears on tissue side.	Isolated fibres.
Protease 6L 16 hours	All epidermis gone.	Slight staining in enamel.	Located near structures but not within destroyed regions.	Small fibres around cells. Not found around destroyed cells.	Separation of bulb from surrounding tissue. Fibronectin appears on tissue side.	Isolated fibres.

Sample / time	Epidermis	Enamel	Grain	Root Sheath	Wool bulb	Corium
Purafect 4000L 16 hours	All epidermis gone.	Slight staining in enamel.	Located near structures but not within destroyed regions.	Small fibres around cells. Not found around destroyed cells.	Separation of bulb from surrounding tissue. Fibronectin appears on tissue side.	Isolated fibres.
Protease 6L 20 hours	All epidermis gone.	Slight staining in enamel.	Located near structures but not within destroyed regions.	Cells mostly destroyed.	Separation of bulb from surrounding tissue. Fibronectin appears on tissue side.	Isolated fibres.
Purafect 4000L 20 hours	All epidermis gone.	Slight staining in enamel.	Located near structures but not within destroyed regions.	Cells mostly destroyed.	Separation of bulb from surrounding tissue. Fibronectin appears on tissue side.	Isolated fibres.
Protease 6L pickled pelt	None.	Visible within enamel.	Located near structures but not within destroyed regions.	None.	Separation of bulb from surrounding tissue. Fibronectin appears on tissue side.	None.
Purafect 4000L pickled pelt	None.	Visible within enamel.	Located near structures but not within destroyed regions.	None.	Separation of bulb from surrounding tissue. Fibronectin appears on tissue side	None

5.4.4.2 Changes to elastin during enzymatic depilation

Previous results have indicated that the persistence of elastin during processing is affected by enzymes. While it was not found in the regions that might be relevant to enzyme depilation, such as the cells of the epidermis and wool root sheaths, its destruction by enzymes has been shown to detrimentally affect the quality of the pickled pelt. Therefore the progress of its destruction during enzyme depilation was of interest.

The results for the full set of samples from the enzymatically depilated skins are described in Table 24.

Table 24: Immunohistology of elastin in enzymatically depilated pelts.

Sample time	Epidermis	Enamel	Grain	Root Sheath	Wool bulb	Corium
Green skin	No elastin observed within epidermis.	Difficult to observe specific staining in green skin within enamel.	Fibres appear throughout grain with a horizontal arrangement in upper grain but randomly arranged in the lower grain. Concentrated around macro structures.	Elastin projects from the outer edge of the ORS into the grain. No elastin is visible within the root sheath.	Elastin projects from the outer edge of the bulb into the grain. No elastin is visible within the root sheath.	Scattered through corium in association with collagen bundles.
Protease 6L 4 hours	No elastin observed within epidermis.	Some elastin observed in the enamel projecting into the grain.	Elastin as yet unaffected.	Elastin as yet unaffected.	Elastin as yet unaffected.	Elastin as yet unaffected.
Purafect 4000L 4 hours	No elastin observed within epidermis.	Some elastin observed in the enamel projecting into grain.	Mostly broken fibres that are now disordered.	Elastin as yet unaffected.	Elastin as yet unaffected.	Elastin as yet unaffected.
Protease 6L 8 hours	No elastin observed within epidermis.	A few fibres remain as observed at 4 hours. Elastin mostly gone.	Mostly broken fibres that are now disordered. Upper grain fibres mostly gone.	Cells of root sheath collapsed or collapsing. Elastin in upper grain gone, elastin in lower grain intact.	Separation of bulb from surrounding tissue. Elastin appears on tissue side.	Elastin as yet unaffected.
Purafect 4000L 8 hours	No elastin observed within epidermis.	Mostly gone from enamel. Rough enamel surface observed with no associated elastin present.	Only some broken fibres remain.	Cells of root sheath collapsed. Elastin in upper grain gone, elastin in lower grain intact.	Separation of bulb from surrounding tissue. Elastin appears on tissue side.	Elastin as yet unaffected.

Sample time	Epidermis	Enamel	Grain	Root Sheath	Wool bulb	Corium
Protease 6L 16 hours	No elastin observed within epidermis	A few fibres remain as observed at 4 hours. Elastin mostly gone	Only some broken fibres remain.	Cells of roots sheath collapsed. Elastin in upper grain gone, elastin in lower grain intact.	Separation of bulb from surrounding tissue. Elastin appears as broken fragments on tissue side.	No elastin observed.
Purafect 4000L 16 hours	No elastin observed within epidermis	Mostly gone from enamel. Roughening of enamel surface observed and no associated elastin.	Only some broken fibres remain.	Root sheath fully collapsed. Small broken fragments of elastin remain around sheaths	Separation of bulb from surrounding tissue. Elastin appears as broken fragments on tissue side	No elastin observed
Protease 6L 20 hours	No elastin observed within epidermis.	Heavily roughened surface, no elastin staining observed.	Isolated regions contain some broken fibres.	Structure no longer distinguishable.	Scattered fibres appear along the line of break.	No elastin observed.
Purafect 4000L 20 hours	No elastin observed within epidermis.	Intermediary roughening, only traces of elastin fibres remain.	Isolated regions contain some broken fibres.	Structure no longer distinguishable.	Scattered fibres appear along the line of break.	No elastin observed.
Protease 6L pickled pelt	No elastin observed within epidermis.	Some traces of fibrous fragments remain.	Only occasional fragments remain	Structure no longer distinguishable.	Scattered fibres appear along the line of break. Region around the bulb now collapsed and very thin.	No elastin observed.
Purafect 4000L pickled pelt	No elastin observed within epidermis.	Some traces of fibrous fragments remain.	Only occasional fragments remain.	Structure no longer distinguishable.	Scattered fibres appear along the line of break. Region around the bulb now collapsed and very thin.	No elastin observed.

Elastin was degraded during enzymatic depilation. Its presence had previously been found to be important in the conventional processing of skins particularly during the enzyme based process of bating as described in sections 2.6.5.1 and 5.4.3.5. Since elastin was observed in the grain, enamel, and the epidermis, damage to elastin may be correlated to damage to the enamel surface in the crust. Furthermore, damage to the elastin network may result in loose leather which is a fault of conventionally processed skins in which elastin has been degraded (Allsop *et al.*, 2005). Thin elastin fibres, which passed through the enamel, were destroyed by both enzyme treatments, and larger fibres lying horizontally to the enamel were broken up into fragments. Some small fibres penetrated into the root sheaths and surrounded the root sheaths in high concentration, although these only represented a small proportion of the root sheath that was destroyed during depilation. It can be inferred therefore from these data that while destruction of elastin is associated with the depilation process it may not be necessary in order to achieve depilation. It may be that elastolytic acitivity is a side effect of the broad spectrum protease activity which is associated with an active depilatory enzyme. Indeed research to date (Allsop *et al.*, 2005) including that carried out in this study, support the premise that removal of elastin from lamb pelts is detrimental and processing enzymes should be explicitly selected for their lack of activity against elastin.

5.4.4.3 Changes to collagen VI during enzymatic depilation

The location of collagen VI in the grain enamel surface in green skin and during conventional processing suggest that it is an important component of the matrix of undamaged pelts both before and after depilation. It was therefore important to assess the integrity of collagen VI during the enzyme depilation process. The results for this investigation are shown in Table 25:

Collagen VI was only found in the enamel, particularly in the region immediately below the epidermal cells. The enamel of conventionally processed skins showed a smooth surface characterised by even staining with the collagen VI antibodies (Figures: Figure 68,Figure 69,Figure 70, and Figure 71). In contrast, the enamel of skins in the early stages of enzyme depilation showed a surface that was beginning to become frayed and a reduction in staining. Therefore a direct relationship appears to be present between the presence of collagen VI, the integrity of the enamel layer and a smooth surface in the

final product. Furthermore it is worth noting that the damage to collagen VI caused by Purafect was less in comparison to the other enzymes.

Table 25: Immunohistology of collagen VI in enzymatically depilated pelts.

Sample time	Epidermis	Enamel	Grain	Root Sheath	Wool bulb	Corium
Green skin	No collagen VI was observed in the epidermis.	Significant staining throughout the enamel.	No specific staining noted below enamel.	No staining of the root sheath. Some adjacent staining consistent with the enamel.	No specific staining observed within the wool bulb	No specific staining was observed in the corium.
Protease 6L 4 hours	Epidermis intact with no specific staining observed.	Some staining observed in the enamel.	No specific staining noted below enamel.	No specific staining in or around root sheath.	No specific staining observed within the wool bulb.	No specific staining was observed in the corium.
Purafect 4000L 4 hours	Epidermis intact with no specific staining observed.	Significant staining throughout the enamel.	No specific staining noted below enamel.	No staining of root sheath, some adjacent staining consistent with the enamel.	No specific staining observed within the wool bulb.	No specific staining was observed in the corium.
Protease 6L 8 hours	Epidermis destroyed.	Heavy staining throughout enamel. Some stained frayed fragments.	No specific staining noted below enamel.	No staining of root sheath, some adjacent staining consistent with the enamel.	No specific staining observed within the wool bulb.	No specific staining was observed in the corium.
Purafect 4000L 8 hours	Epidermis shedding from the enamel. Some staining visible on both sides of break.	Staining throughout the enamel. Some stained frayed fragments below shed epidermis.	No specific staining noted below enamel.	No staining of root sheath, some adjacent staining consistent with the enamel.	No specific staining observed within the wool bulb.	No specific staining was observed in the corium.

Sample time	Epidermis	Enamel	Grain	Root Sheath	Wool bulb	Corium
Protease 6L 16 hours	Epidermis destroyed.	Some stained fragments remain.	No specific staining noted below enamel.	No staining in root sheath.	No specific staining observed within the wool bulb.	No specific staining was observed in the corium.
Purafect 4000L 16 hours	Epidermis destroyed.	Some stained fragments remain.	No specific staining noted below enamel.	No staining in root sheath.	No specific staining observed within the wool bulb.	No specific staining was observed in the corium.
Protease 6L 20 hours	Epidermis destroyed.	Some stained fragments remain.	No specific staining noted below enamel.	No staining in root sheath.	No specific staining observed within the wool bulb.	No specific staining was observed in the corium.
Purafect 4000L 20 hours	Epidermis destroyed.	Some stained fragments remain.	No specific staining noted below enamel.	No staining in root sheath.	No specific staining observed within the wool bulb.	No specific staining was observed in the corium.
Protease 6L pickled pelt	Epidermis destroyed.	Only isolated fragments remain.	No specific staining noted below enamel.	No staining in root sheath.	No specific staining observed within the wool bulb.	No specific staining was observed in the corium.
Purafect 4000L pickled pelt	Epidermis destroyed.	Only isolated fragments remain.	No specific staining noted below enamel.	No staining in root sheath.	No specific staining observed within the wool bulb.	No specific staining was observed in the corium.

The observation are consistent with the results in section 3.2.1.7 for the screening of enzymes based on overall depilatory effectiveness and the least damage. That experiment also identified Purafect as the best performing enzyme.

5.4.4.4 Changes to collagen VII during enzymatic depilation

Table 26: Immunohistology of collagen VII in enzymatically depilated pelts.

Sample time	Epidermis	Enamel	Grain	Root Sheath	Wool bulb	Corium
Green skin	No collagen VII was observed in the epidermis.	Only very rarely was any specific staining observed in the enamel.	The grain was studded with many irregular angular plaques around 6 um in diameter.	No staining was observed in the root sheath.	The wool bulb was heavily stained and it was difficult to discern specific or non specific staining.	No specific staining was observed in the corium.
Protease 6L 4 hours	Epidermis intact with no specific staining observed.	Only very rarely was any specific staining observed in the enamel.	The grain was studded with many irregular angular plaques around 6 um in diameter.	No staining was observed in the root sheath.	In addition to some angular plaques there was some amorphous staining was observed within the bulb.	No specific staining was observed in the corium.
Purafect 4000L 4 hours	Epidermis intact with no specific staining observed.	Only very rarely was any specific staining observed in the enamel.	The grain was studded with many irregular angular plaques around 6 um in diameter.	No staining was observed in the root sheath.	In addition to some angular plaques there was some amorphous staining was observed within the bulb.	No specific staining was observed in the corium.
Protease 6L 8 hours	Epidermis collapsed with no specific staining observed.	Only very rarely was any specific staining observed in the enamel.	The grain was studded with many irregular angular plaques around 6 um in diameter.	No staining was observed in the root sheath.	In addition to some angular plaques there was some amorphous staining was observed within the bulb.	No specific staining was observed in the corium.
Purafect 4000L 8 hours	Epidermis collapsed with no specific staining observed.	Only very rarely was any specific staining observed in the enamel.	The grain was studded with many irregular angular plaques around 6 um in diameter. Much less appear in the upper grain in comparison to the lower grain.	No staining was observed in the root sheath.	In addition to some angular plaques there was some amorphous staining was observed within the bulb.	No specific staining was observed in the corium.

Sample time	Epidermis	Enamel	Grain	Root Sheath	Wool bulb	Corium
Protease 6L 16 hours	Epidermis gone.	Only very rarely was any specific staining observed in the enamel.	Studded with scattered plaques but fewer in number than observed in previous slides.	No staining was observed in the root sheath.	In addition to some angular plaques present, some amorphous staining within the bulb was observed.	No specific staining was observed in the corium.
Purafect 4000L 16 hours	Epidermis gone.	Only very rarely was any specific staining observed in the enamel.	Studded with scattered plaques but fewer in number than observed in previous slides.	No staining was observed in the root sheath.	A few angular plaques remain. No amorphous staining observed.	No specific staining was observed in the corium.
Protease 6L 20 hours	Epidermis gone.	No staining observed.	Very rare isolated thin plaques.	No staining was observed in the root sheath.	A few angular plaques remain. No amorphous staining observed.	No specific staining was observed in the corium.
Purafect 4000L 20 hours	Epidermis gone.	No staining observed.	Very rare isolated thin plaques.	No staining was observed in the root sheath.	A few angular plaques remain. No amorphous staining observed.	No specific staining was observed in the corium.
Protease 6L pickled pelt	Epidermis gone.	No staining observed.	Very rare isolated thin plaques.	No staining was observed in the root sheath.	A few angular plaques remain. No amorphous staining observed.	No specific staining was observed in the corium.
Purafect 4000L pickled pelt	Epidermis gone.	No staining observed.	Very rare isolated thin plaques.	No staining was observed in the root sheath.	A few angular plaques remain. No amorphous staining observed.	No specific staining was observed in the corium.

While collagen VII did not appear to be associated with the regions linked to enzymatic depilation or the damage that occurs during enzymatic depilation, it was interesting to include this analysis and to assess the presence of this protein in the skins during the enzymatic depilation process.

Collagen VII did not appear in the epidermis or root sheaths so the loosening of the wool fibre during enzymatic depilation that resulted as wool sheath was destroyed is not likely to be due to the destruction of collagen VII. Some staining was observed, however, within the wool root bulb, indicating the presence of collagen VII. It is possible that the destruction of collagen VII within the wool bulb may assist in the depilation process. However, the staining remained during the depilation process and the structures that were stained within the wool root remained intact until well after depilation. Depilation cannot therefore be associated with the destruction of collagen VII. Nevertheless, collagen VII was damaged during the depilation process as illustrated by its disappearance from the upper grain. As collagen VII was not observed in the grain enamel it can be concluded that the destruction of this protein was not the cause of grain surface damage observed during the depilation process. In conclusion, collagen VII does not appear to play an important role in the enzyme depilation process and a collagen VII specific protease would be unlikely to have a positive affect on the depilation process.

5.4.4.5 Response of laminin during enzymatic depilation

Since laminin was found in the root sheath of conventionally depilated pelts it was important to attempt to identify the importance of laminin during enzymatic depilation. The results of the immuno histology for laminin are given in Table 27.

The removal of laminin during enzymatic processing appeared to coincide with destruction of material associated with wool root sheath loosening. As the structures of the wool root sheaths were destroyed so the staining for laminin was reduced. Laminin did not appear to be associated with the enamel surface and is unlikely to be associated with enzyme damage. However because it is associated with the wool root sheath, laminin is a good target for specific enzyme depilation.

Table 27: Immunohistology of laminin in enzymatically depilated pelts

Sample time	Epidermis	Enamel	Grain	Root Sheath	Wool bulb	Corium
Green skin	No positive staining observed.	No positive staining observed.	No positive staining observed.	Some positive staining observed around cells of inner root sheath.	Some positive staining observed around cells of at the outer edge of wool bulb.	No positive staining observed.
Protease 6L 4 hours	No positive staining observed.	No positive staining observed.	No positive staining observed.	Some positive staining observed around cells of inner root sheath.	Some positive staining observed around cells of at the outer edge of wool bulb.	No positive staining observed.
Purafect 4000L 4 hours	No positive staining observed.	No positive staining observed.	No positive staining observed.	Some positive staining observed around cells of inner root sheath.	Some positive staining observed around cells of at the outer edge of wool bulb.	No positive staining observed.
Protease 6L 8 hours	Most epidermis gone.	Some positive staining in pieces at epidermal edge.	No positive staining observed.	Some positive staining observed around cells of inner root sheath.	Separation of bulb from surrounding tissue. Laminin appears on wool side.	No positive staining observed.
Purafect 4000L 8 hours	Most epidermis gone.	Some positive staining in pieces at epidermal edge.	No positive staining observed.	Some positive staining observed around cells of inner root sheath.	Separation of bulb from surrounding tissue. Laminin appears on wool side.	No positive staining observed.
Protease 6L 16 hours	All epidermis gone.	No positive staining observed.	No positive staining observed.	Some positive staining observed around the cells of the inner root sheath.	Positive staining in the wool bulb except for the dermal papilla which remains unstained.	No positive staining observed.
Purafect 4000L 16 hours	All epidermis gone.	No positive staining observed.	No positive staining observed.	Some positive staining observed around the cells of the inner root sheath.	Positive staining in the wool bulb except for the dermal papilla which remains unstained.	No positive staining observed.

Sample time	Epidermis	Enamel	Grain	Root Sheath	Wool bulb	Corium
Protease 6L 20 hours	All epidermis gone.	No positive staining observed.	No positive staining observed	Some positive staining observed around the cells of the inner root sheath.	The wool bulb is now broken up but positive staining persists in the wool bulb region.	No positive staining observed.
Purafect 4000L 20 hours	All epidermis gone.	No positive staining observed.	No positive staining observed.	Some positive staining observed around the cells of the inner root sheath.	The wool bulb is now broken up but positive staining persists in the wool bulb region.	No positive staining observed.
Protease 6L pickled pelt	None.	No positive staining observed.	No positive staining observed.	No positive staining observed.	The wool bulb is now broken up but positive staining persists in the wool bulb region.	No positive staining observed.
Purafect 4000L pickled pelt	None.	No positive staining observed.	No positive staining observed.	No positive staining observed.	The wool bulb is now broken up but positive staining persists in the wool bulb region.	No positive staining observed.

Table 28: Immunohistology of laminin in enzymatically depilated pelts.

5.4.5 Examination of specificity of immunohistology by Western blotting

While some of the immunostaining results pointed to proteins that might be targets for specific enzymes to achieve depilation, collagen VI was the only protein examined which appeared have a possible association with grain enamel damage. When examined using immunohistology the only positive staining that was observed within the enamel layer was when probing for collagen VI.

It is important therefore to determine if the identification of collagen VI in the enamel was definitive. There is a possibility that cross reactivity to other proteins in the skin may have generated the positive staining observed in the grain enamel. While the product description for the collagen VI antibodies (Chemicon AB7822) states that there is less than 1% cross reactivity with human collagen types I, II, III, IV, and V it was prudent to investigate the possibility of cross reactivity with ovine skin proteins using

Western blotting. By preparing a protein extract from raw skin, separating the protein complement from the extract using SDS-PAGE, examining the result using Western blotting and probing with the collagen VI antibody, it might be possible to confirm the presence of only one antigenic protein in raw skin, thereby substantiating the use of the antibody in immunohistology as a means to validate the presence of collagen VI in the enamel.

Protein samples were first extracted and separated on SDS-PAGE then transferred to a blotting membrane and probed with the antibodies. The details of sample preparation and methodology are given below:

5.4.5.1 Sample preparation for electrophoresis method

Skin extract was prepared from raw skin using the method described in section 8.6.1. Briefly; the skin samples were cut into small pieces, immersed in liquid nitrogen then ground using a mortar and pestle and the resulting powder stored at -80 °C for further use. The powdered samples were then suspended in 5 volumes of lysis solution (section 8.5.1), containing 8M urea, 2M thiourea, 4% CHAPS, 20 mM DTT and Complete Mini^{TM} (wide spectrum protease inhibitor at the concentration recommended by the manufacturer) in 40 mM Tris buffer (pH 7.0) for 1 h at room temperature The method used was based on that described in (Berkelman & Stenstedt, 1998) with the use of CHAPs and thiourea also described by (Mujahid *et al.*, 2007) for improved solublization. After this time they were briefly sonicated (Cole-Palmer, model 8891), taking care not to heat the samples, then centrifuged for 15 min at 7500g to remove any insoluble material, and the supernatant collected and stored at –80 °C for further studies (Berkelman & Stenstedt, 1998).

Prior to determination of their protein concentration, the samples were dialysed against Tris-HCl buffer (pH 7.0) to remove any chaotropic agents and detergents which might interfere with the Bradford reagent. Protein concentrations were then determined using the Bradford method, with BSA as the standard (Bradford, 1976).

5.4.5.2 Western blotting method

7% SDS poly acrylamide gels were prepared using the methods described in section 8.4 The method for Western carried out was that described using the Bio-Rad mini-transblot

module (GE Healthcare UK Limited, 2006) using the same blocking concentrations, dilutions and antibody concentrations as used in the immunohistology work as described in section 5.4.1. The method used is described in detail in section 8.7.4.

Immediately after running, the SDS gel was dipped into transfer buffer (0.025 M Tris/glycine-HCl/15% (v/v) Isopropanol, pH 8.3 see section 8.7.4.2), then laid flat on a nitrocellulose membrane (BioRad cat# 162-0116 0.45 um)that had been pre-wetted with transfer buffer and which was supported by pieces of pre-wetted Whatman No. 54 filter paper cut to size, piled in a stack approximately 3mm high resting on the filter pad and then on the anode. The gel was overlaid with another 3mm thick stack of pre-wetted Whatman filter paper and then another filter pad. Care was taken to exclude bubbles between the gel and the nitrocellulose membrane The completed apparatus was then assembled with the membrane on the anode side of the sandwich as shown in Figure 84.

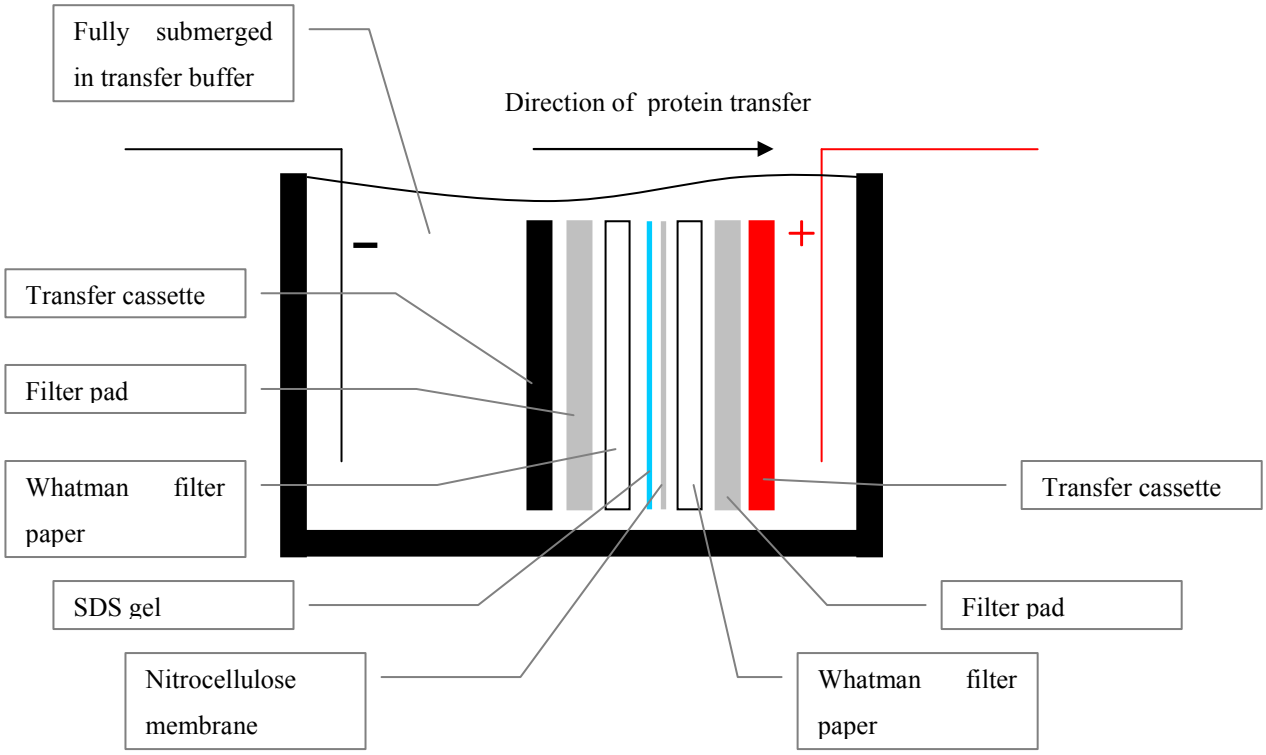


Figure 84: Western blot apparatus set-up of electro blotting sandwich

The apparatus was subjected to 100V for 1 hour and the transfer checked by the transfer of coloured protein markers from the gel to the membrane. The time for transfer was adjusted if necessary. After the proteins had been transferred onto the nitrocellulose paper, the paper was washed and treated to the immunochemical detection procedure as described in section 8.7.4.7.

The biotin-streptavidin detection system was used as follows: The membrane was blocked with 1% BSA in phosphate buffered saline for Western blotting, (1% BSA in 0.01 M Phosphate NaCl 9.0 g/L, 0.05% w/v Tween20) (1% BSA in PBS_W), pH 7.4 with gentle shaking for 1 h. After this time it was washed in three changes of PBS_W (0.01 M Phosphate NaCl 9.0 g/L, 0.05% w/v Tween20) for 5 min each. The membrane was then exposed to 10 mL of anti-collagen VI antibodies (Anti-collagen VI raised in rabbit (Chemicon AB7822) diluted 1:3333 in PBS_W for 1 h with gentle shaking at room temperature. After this time the solution was drained and the membrane washed in three separate changes of PBS_W for 5 min with gentle shaking. It was then exposed to approximately 10 mL Biotinylated IgG (Amersham anti-rabbit from sheep (RPN1004) diluted 1:3333 in 1% BSA in PBS_W) while being gently shaken at room temperature for 1 hr. It was then drained and washed in three changes of PBS_W before being exposed to a preformed biotin-streptavidin-peroxidase complex (Streptavidin biotinylated horseradish peroxidase RPN1051 diluted 1:3333 in 1% BSA in PBS_W). Sections were then drained and washed in three changes of PBS_W before carrying out the detection protocol.

Peroxidase detection was carried out using the ECL method as described in section 8.7.4.8. Briefly, the nitrocellulose membrane was exposed to the chemiluminescence substrate (premixed 10mL substrate A 1000 uL substrate B Roche cat. 1500694) for 1 min and the development of bands monitored using a Fujifilm Intelligent darkbox II.

5.4.5.3 Western blotting results

A sample of pure collagen VI (collagen type VI human adult cartilage, RDI Division of Fitzgerald Industries, RDI-9001108) was used as a positive control. 10 uL of protein (0.5 Mg/mL) was run on a 7-14% gradient gel and tested as above. The results of the Western blot are shown in Figure 85.

A protein extract of raw skin was generated using the method described in section 8.6.1. A sample of mixed collagen was also obtained and examined (Calbiochem – collagen calf skin Lot B38977). In order to determine appropriate concentrations of this extract to be used for the Western blots, different concentrations of the samples were analysed by SDS PAGE.

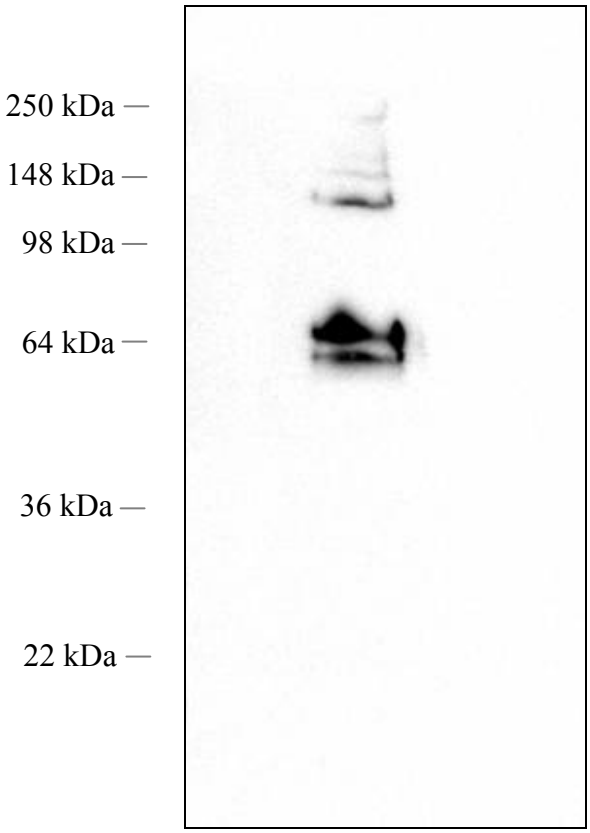


Figure 85: Western blot ECL detection of collagen VI. Using anti-collagen VI raised in rabbit (Chemicon AB7822). Pre-stained markers were used to define the molecular weight distribution (Blue ® plus L pre-stained standard cat LC5925, invitrogen). Different bands represent different alpha chains including the highly glycosilated alpha3 chain.

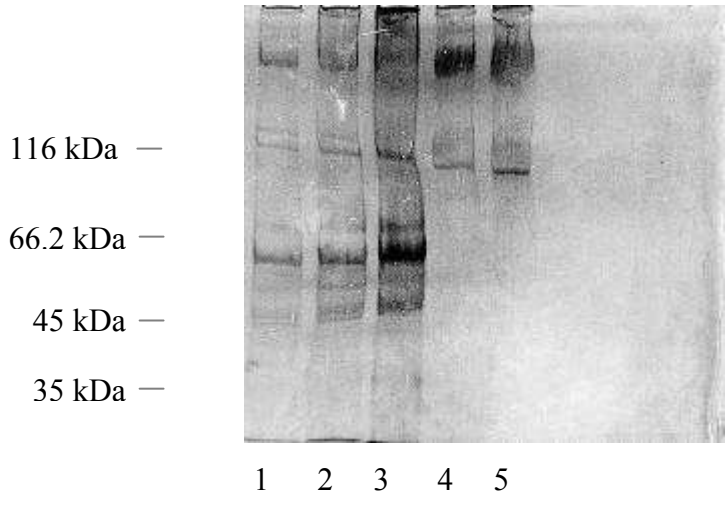


Figure 86: 7% SDS PAGE gel of skin extracts stained with coomassie blue. Unstained markers were used to define the molecular weight distribution (Fermentas #SM0430). Lanes 1,2 and 3 contain 1,2, and 4 uL of raw skin extract respectively,. Lanes 4 and 5 contain 2.5 uL and 5 uL of mixed collagen respectively.

The gel was stained with coomassie blue R-250 (section 8.7.3) It was found that a sample size of 4uL of raw skin extract and 5 uL mixed collagen was appropriate for blotting. Next a gel was run with duplicate lanes of each of the proteins to be examined. Before staining the gel was cut between the 5th and 6th lanes to generate two duplicate gels one of which was stained with coomassie blue R-250 (Figure 87a) and the other blotted (Figure 87b).

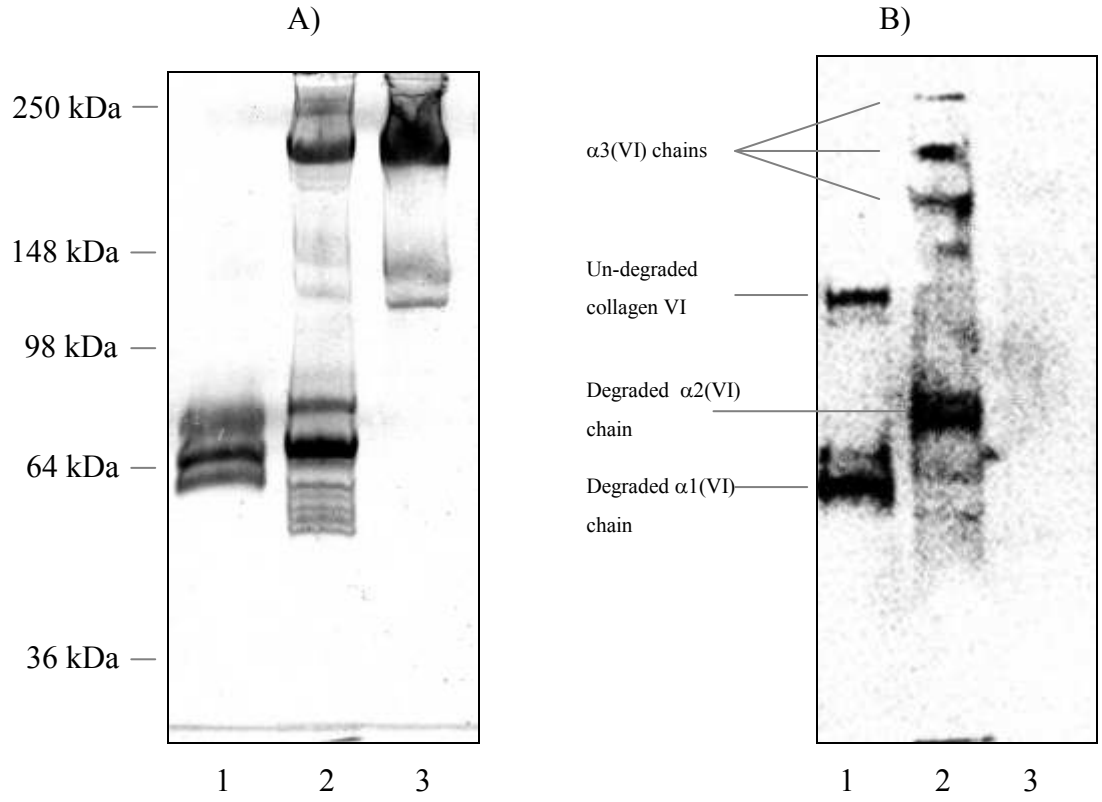


Figure 87: SDS PAGE and Western blot of skin proteins 7.5% Polyacrylamide gel. Pre-stained markers were used to define the molecular weight distribution (Blue ® plus L pre-stained standard cat LC5925, invitrogen). A) Coomassie brilliant blue R-250 stain of SDSPAGE gel. B) Western blot. Lanes in both gels were loaded as follows: 1) Collagen VI, 2) Raw skin extract, 3) mixed collagen.

The results showed that proteins extracted from raw ovine skin did contain collagen VI. The α1(VI) and α2(VI) chains of Collagen VI have previously been shown to present bands at 140kDa on SDS PAGE under reducing conditions ((Wegrowski *et al.*, 1990)) while the α3(VI) chain as suggested to be responsible for bands ranging from 180-240kDa. Proteinase digestion of the α1(VI) and α2(VI) collagen VI glycoproteins resulted in fragments at 55kDa and 65kDa respectively ((Wegrowski *et al.*, 1990)). It is

therefore possible that the bands observed in Figure 87a and b lane 1 represent the degradation products of collagen VI in the collagen VI standard used, that the band observed at about 140kDa in Figure 87b represents the un-degraded α1(VI) or α2(VI) chains from the standard. Furthermore it is possible that the higher molecular weight bands observed in Figure 87b lane 3 represent bands from the α3(VI) chain as observed by (Wegrowski *et al.*, 1990), and the band at about 65kDa in Figure 87b lane 3 represents a degradation product of the collagen VI α2(VI) chain.

No bands were observed in the mixed collagen after blotting (Figure 87b) despite good bands on the SDSPAGE gel (Figure 87a) further indicating that the antibodies were specific to collagen VI. It would be useful however to confirm these results using an alternative method.

In summary, Western blots showed that collagen VI antibodies were specific for collagen VI and therefore the immunohistology results (sections 5.4.3.2, 5.4.4.3) are likely to be meaningful.

5.4.6 Individual protein changes during depilation - Summary

Fibronectin was found to be located within the epidermis rather than in or around the root sheath and therefore is unlikely to play a role in anchoring the wool to the skin. Laminin on the other hand was located near the regions of the skin that were destroyed during enzymatic depilation and did not appear to be associated with the grain enamel. Laminin therefore is a promising target for enzyme depilation.

The destruction of elastin does not appear to relate to the depilation process but may be related to looseness in enzymatically depilated skin which might occur during enzyme depilation. As elastin is also present in the grain enamel in the form of vertical fibres, its destruction might be a factor in surface damage observed in enzymatic depilation. However, since the elastin is also removed from the enamel of conventionally processed skin this is unlikely.

Collagen VII was visualised as small plaques and did not appear to be associated with either the depilation mechanism or with the process of damage to the enzymatically depilated skin enamel.

Using the techniques and the antibodies that were available, it was not possible to assess the importance of collagen III, collagen IV, or collagen V in enzyme depilation. It is possible that further examination of these proteins using alternative sources of antibodies or ovine-specific antibodies may elucidate the relevance of these minor proteins.

Collagen VI was examined in this work and immunohistology indicated that it was found in the enamel of conventionally processed skins but not the enamel of enzymatically depilated skins. This is important in that it indicates that the removal of collagen VI may be associated with the damage to the enamel observed in enzymatically depilated skins. As an alternative to immunohistochemistry it was thought that 2D electrophoresis might be a useful method for investigating changes in the protein complement of skin during the enzymatic depilation process and to confirm the importance of collagen VI.

5.5 Determining the reason for damage to surface proteins

Immunohistochemistry results suggested that removal of minor protein components, particularly Collagen VI could be a principle mechanism of grain enamel layer damage. Because avoidance of damage in this layer is the key to development of a viable enzyme depilation process, further work on characterising the surface structure and composition of the skin was required

Previous work aimed at determining the nature of this surface has been limited mostly to optical and scanning electron microscopy (Haines, 1984b); (Dempsey, 1984); (Stirtz, 1965). These studies showed that the enamel has a different collagen morphology to that of the rest of the grain layer below it (Dempsey, 1984) (Bienkiewicz, 1983). In particular the distribution of the diameter of the fibrils in the enamel and the grain layers was shown to be significantly smaller (Stewart, 1995).

Previous iso-electric focusing experiments have shown that proteins from pickled pelt focus in the pH range of 4 – 7, and that the protein complement of the raw skin is severely depleted after conventional processing (Deb Choudhury *et al.*, 2006).

This section of work focuses on the fine structure and composition of the enamel layer relative to that of the remainder of the grain layer. The strategy was to characterise the nature of the grain enamel layer, to determine the differences in the protein composition of this layer from skins subjected to different processing regimes, and to determine the effect of the different regimes on the proteins in this layer. In this way quantitative data on the surface composition data can potentially validate the qualitative results found using immunohistochemistry. The advantage of proteomic techniques to achieve this is clear as illustrated by their recent use to investigate the changes in protein complement during oxidative unhairing (Marmer & Dudley, 2007) and investigation into enzymatic changes induced in gelatine (Taylor *et al.*, 2006).

5.5.1 Surface protein investigation method

Since collagens are the major component of the pickled pelt they dominate the sample in any type of analysis, making it difficult to detect differences in the minor components. In order to determine whether there are any differences in the populations of minor proteins between the enzymatic and conventionally depilated skins these minor proteins need to be enriched. However, it is difficult to remove one component of a complex mixture of proteins without the loss of others. Another approach is to isolate the layer of the skin of interest, on the assumption that the specific proteins important for its integrity will be present in higher concentrations (von Loutis *et al.*, 1984). The approach taken here was to physically sample the layer of interest rather than chemically separate all the proteins in order to identify the unmodified protein complement of the layer.

5.5.1.1 Surface protein investigation reagents

A number of reagents were sourced for the protein assays used in this work. Tris(hydroxymethyl)aminomethane,3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), thiourea, urea, iodoacetamide (IAM), sodium dodecyl sulfate (SDS), N-ethylmaleimide, α–cyano-4-hydroxy-trans-cinnamic acid,

dithiothreitol (DTT), and diaminobenzidine were purchased from Sigma. pH 4-7 carrier ampholytes, biotinylated antibody raised against rabbit, and biotin-streptavidin-peroxidase complex were obtained from Amersham Biosciences. Sequence-grade modified porcine trypsin was obtained from Promega. Phenylmethylsulfonyl fluoride (PMSF), n-(2-hydroxyethyl) piperazine-n′-(2-ethanesulfonic acid) (HEPES), sodium carbonate, sodium bicarbonate, and sodium dihydrogen orthophosphate were sourced from BDH Chemicals Ltd (Poole, England). Complete Mini^{TM} was obtained from Roche Diagnostics, Mayers Hämalaun solution from Applichem GmbH, Darmstadt, Germany, DPX mounting medium from (Labchem, Australia), and 30% Peroxide solution from Scharlau chemie, Spain. Acetonitrile (MeCN), butanol and dichloromethane were obtained from SDS chemicals (France). Tanzyme was sourced from Tryptec Biochemicals, (New Zealand), ammonium sulfate from Clark Products, (New Zealand), hydrated lime from Websters Hydrated Lime Co. Ltd, (New Zealand), and starch thickener from Solvitose, Avebe, (Holland). All other chemicals were all of analytical grade.

5.5.1.2 Initial sample generation

Green skins were obtained and prefleshed prior to processing. A skin was divided into three portions designated; green, conventional and enzyme. The “green” skin was sampled immediately, the “conventional” was processed through to pickled pelt using the LASRA procedure (see appendix section 8.1). and the “enzyme” skin was treated as described in section 3.2.1 Briefly, the skins were exposed to 4 volumes of enzyme buffer containing 2% Purafect 4000L (v/v), 0.5 M Sodium carbonate/bicarbonate buffer at pH 10.5, 0.2% potassium dimethyldithiocarbamate solution (Busan 85, Buckmans: Australia) - 30%w/v active, and 0.5% Teric BL8 (Huntsman: Australia). After 30 minutes the skins were squeezed dry using a mangle to remove excess depilatory and then held at 20°C for 16 hours. The skins were then pickled with the standard pickle process (Appendix 8.2.3) with an additional 1%w/v formic acid added to neutralise the carbonate buffer.

Square pieces of skin approximately 5 by 5 mm were excised from the official sampling position ((IUP 2, 2000)) of the differently processed half skins and placed flat on a cryostat stub. Frozen samples of skin 20 um thick were cut horizontally from the upper (grain) surface with a cryostat (Jung Frigocut 2800E, Leica) and examined under a

binocular Nikon Optishot microscope at 50X magnification. The sections were designated as being either the grain enamel layer or the remaining grain layer according to their appearance. The first four 20 um sections cut from the upper surface of each sample were designated “enamel”. The next six sections contained significant amounts of both the enamel and the remaining grain layers as illustrated in Figure 88 and so were discarded. The following six sections were collected and designated “remaining grain layer”. These samples were then prepared for 2D electrophoresis. A second set of excised skin samples was prepared and the uppermost section of the enamel layer was fixed to a slide and examined using atomic force microscopy.

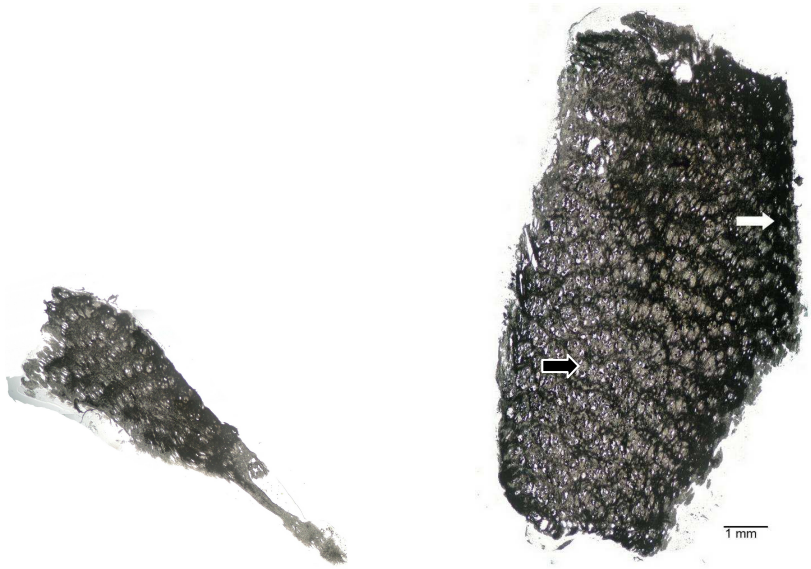


Figure 88: Stratified samples. Left - 1st section cut from surface. Right 6th section cut from surface containing regions of both enamel layer and the remainder of the grain layer. The enamel layer is visible as the darker region (white arrow) around the outside of the section particularly at the top right of the image. The lighter region (dark arrow) is the remainder of the grain layer that has been uncovered by the removal of successive sections.

The two sections were quite different in that the material close to the outside edge of the skin surface was darker when viewed under transmission microscopy.

It is clear that there was a difference in structure between the papillary layer and the reticular layer which can be distinctly observed in the sample from the 6^{th} cut (representing 120 um depth). For the preparation of samples for 2D electrophoresis samples were collected for the papillary layer from only the first 4 sections, a further 10

section were cut to remove the remainder of the papillary layer and then the next four sections were collected for the proteins of the remaining grain layer.

The samples were then stored at –80°C until required for 2D electrophoresis. Samples for electrophoresis were prepared using the method described in detail in section 5.4.5.1.

5.5.1.3 Atomic force microscopy method

It has been suggested previously that the use of scanning force microscopy is a useful technique for the investigation of changes in surface character of leather (Reich *et al.*, . An atomic force microscope, (MFP-3D Asylum Research, Santa Barbara. CA) was therefore used to study the differences in the surface topology of skins depilated using conventional methods or enzymes. Silicon cantilevers, (Ultrasharp CSG-11, NT-MDT, Zelenograd, Moscow, Russia) were used to study the samples in contact mode. The tip, nominally 10 nm in radius, was located on the free end of a cantilever 350 um long, 35 um wide and 0.7-1.3 um thick with a nominal force constant of 0.01-0.08 N/m. A tip speed of 40 um/s was used to scan the samples.

5.5.1.4 Electrophoresis method

Isoelectric focusing was carried out at 20 °C using an IPGphor unit (Amersham Biosciences). Pickled skin extracts (50 uL) (generated from samples prepared as described in section 5.5.1.2 and extracted as described in section 5.4.5.1) having total protein concentrations of approximately 0.1 mg/mL, were solubilised by incubation for 30 min in 125 uL of rehydration solution, (2M thiourea, 8M urea, 20mM DTT, 0.5% v/v IPG buffer, pH 4-7, a trace of Bromophenol blue, 2% CHAPS and Complete Mini^{TM}). The solution was then centrifuged at 11,300g for 2 min and the clear supernatant was applied to 7 cm IPG strips (Amersham Biosciences), pH 3-10. The process was repeated for the longer IPG strips (18cm) over a narrower pH range, (pH 4-7). For these strips, the protein concentration was approximately 0.3 mg/mL, the IPG buffer used was pH 4-7 and the volume of rehydration buffer used, 340 uL. Active rehydration of the strips was carried out at 30 Volts (V) for 10 h, followed by isoelectric focusing using the following programs:

a) For 7 cm strips: 300 V step'n hold for 3 h, 1000 V gradient for 30 min, 5000 V gradient for 90 min and a final focusing step at 5000 V step'n hold for 3 h.

b) For 18 cm strips: 500 V step'n hold for 1 h, 1000 V gradient 1 h, 8000 V gradient 3 h, 8000 V step'n hold for 3 h.

The focused IPG strips were then equilibrated in two volumes of equilibration buffer (50mM Tris buffer, pH 8.8, containing 6M urea, 30% w/v glycerol, 2% w/v SDS and a trace of bromophenol blue); 10 mL containing 10 mg/mL DTT, for 15 min followed by 10 mL containing 25 mg/mL iodoacetamide for a further 15 min. The second dimension electrophoresis was carried out either on a BioRad Mini-PROTEAN® 3 system for 7 cm strips or BioRad PROTEAN II xi system for the 18 cm strips. Acrylamide gels (0.75 mm thick, 7.5% acrylamide) were cast without wells and the IPG strips laid gently on top of the gels then sealed in place with 1% agarose. Electrophoresis was carried out at 100 V for 15 min, and then increased to a constant 200 V until the end of the run.

5.5.1.5 Preparation for matrix assisted laser desorption ionisation time of flight analysis

The protein spots were excised from the stained gel and cut into small pieces of about 1x1 mm on a clean glass surface using a clean razor blade, then transferred to Eppendorf tubes and washed with several changes of 25 MM NH_4HCO_3 followed by several changes of water. They were then suspended in 1% (v/v) formic acid before being dehydrated by exposing them to a solution of 50% (v/v) water, 50% (v/v) Acetonitrile, made 1% (v/v) in formic acid, for 5 min then finally with 100% acetonitrile for 10 min. To generate a peptide fingerprint in-gel tryptic digestion was performed. (Shevchenko *et al.*, 1996). Briefly, the Eppendorf tubes containing the gel slices were placed in an ice water bath and the gel particles were allowed to swell in 25-35 uL digestion buffer containing 12.5 ng/uL of sequencing grade trypsin in 50 mM NH_4HCO_3 for 45 min. The trypsin containing buffer was then removed and 5-10 uL of 50 mM NH_4HCO_3 was added to keep the pieces wet during an overnight incubation at 37 °C. After incubation the supernatant was separated from the gel pieces by centrifugation at 11300g and saved in a separate Eppendorf tube; the gel pieces were re-extracted with NH_4HCO_3 (20 uL of a 20mM solution) for 10 min, and the supernatant

pooled with that from the first extraction. The gel pieces were then re-extracted with 25 uL of 5% formic acid and 50% acetonitrile for 20 min, centrifuged at 11300g and this supernatant pooled with those from the previous steps. The formic acid extraction was repeated twice to give a final extraction volume of ~105 uL, which was reduced using evaporation under vacuum (Savant Speed-vac, SC-100).

5.5.1.6 MALDI TOF analysis method

The rapid evaporation method was used for the sample preparation for MALDI-TOF analysis (Shevchenko *et al.*, 1996). Briefly 1 uL of peptide matrix/nitrocellulose solution (10 mg nitrocellulose and 40 mg α–cyano-4-hydroxy-trans-cinnamic acid in 1mL acetone and 1mL isopropanol that had been freshly prepared) was applied to the sample target. 2 uL of peptide solution was then concentrated and desalted using C18 ZipTips (Millipore Corporation. Bedford, U.S.A.) according to the manufacturer's instructions. One uL of the peptide eluate from the ZipTip was then directly pipetted on to the matrix solution and the sample co-crystallised with the matrix by evaporating to dryness at ambient temperature. The organic solvent in the sample was kept below 10% to ensure that the sample solution did not completely dissolve the matrix layer. The sample target was inserted into the MALDI-TOF mass spectrometer (M@LDI^{TM} Micromass) and analysed in positive ion reflectron mode.

5.5.2 Results

In this work an attempt was made to analyse the protein constituents and structure of the enamel, the most important part of the skin as far as its appearance is concerned, in order to determine whether the removal of specific proteins is responsible for the differences observed in the products from conventionally processed and enzymatically depilated skins.

5.5.2.1 Atomic force microscopy

Using atomic force microscopy it was possible to view the changes in the leather surface that occurred when skins were processed using either enzymes or the conventional sulfide based process. The enamel layers of conventionally processed and an enzyme depilated skin are shown in Figure 89 and Figure 91 respectively where differences in their surface topographies are quite evident.

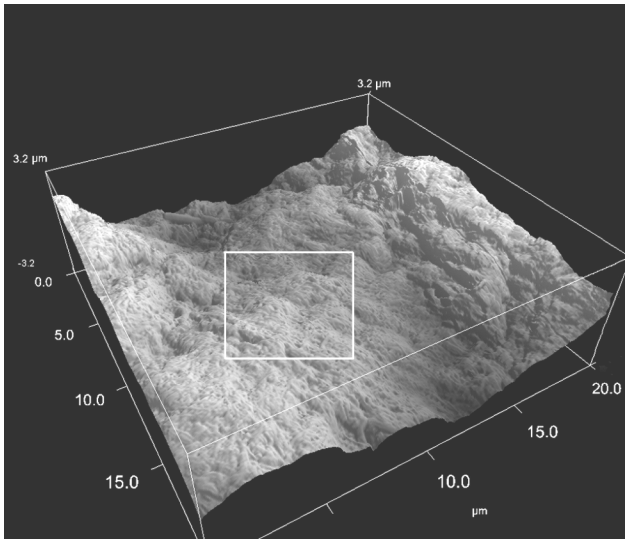


Figure 89: AFM image of outermost surface of conventionally depilated pickled lambskin scanned at 20 um resolution. The collagen is visible as single fibrils lying in a fine mesh. The box marked in white is magnified in Figure 90.

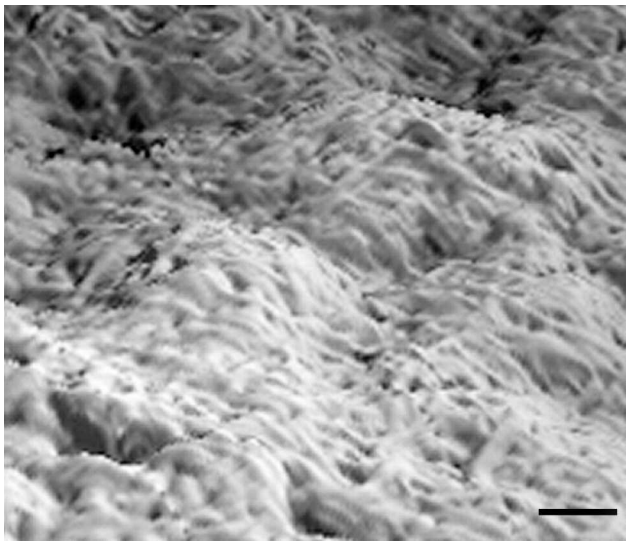


Figure 90: Magnification of the AFM image of conventionally depilated skin shown in Figure 89 showing detail of the fine mesh of individual collagen fibrils. The bar in the magnified region is 1 um

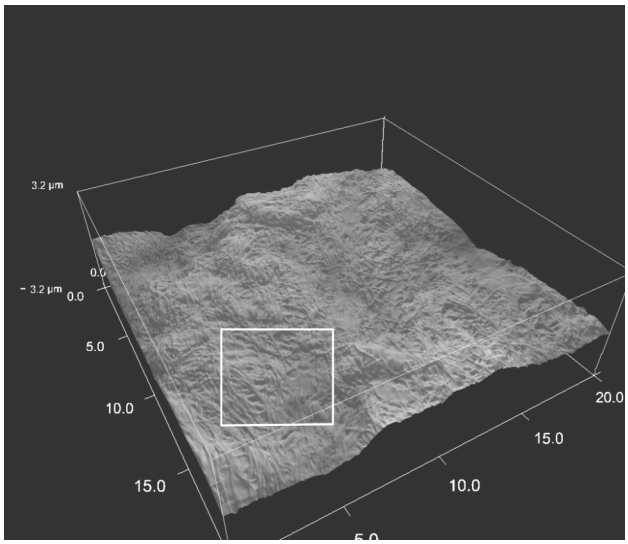


Figure 91: AFM image of outermost surface of enzymatically depilated pickled lambskin scanned at 20 um. The collagen is visible as single fibrils lying in a disorganised pattern. Some fibrils lie enmeshed with others but a significant number appear loose and independent of the rest of the surface structure. The box marked in white is magnified in Figure 92.

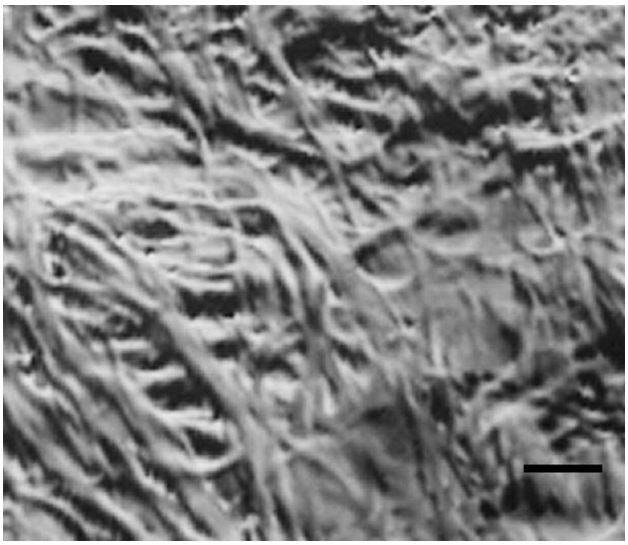


Figure 92: Magnification of the AFM image of enzymatically depilated skin shown in Figure 91 showing detail of the disorganised loose mesh of collagen fibrils. The bar in the magnified region is 1 um

The enamel layer of the conventionally treated material shows a fine, tightly interwoven mesh of individual collagen fibrils lying at right angles to one another which can be seen in more detail in Figure 90. In contrast, the individual fibres at the surface of the enzyme-depilated material appear to be disentangled from that fine mesh and can be seen lying free (Figure 91). Furthermore the woven mesh structure appears to be much

looser and more open than the conventionally treated material. This is particularly evident in Figure 92 (a magnification of Figure 91).

The fine structure observed here on ovine material differs from that observed with deer, cattle, and pigs which exhibit a fine structure made up of some single fibrils but also significant groups of a few collagen fibrils tangled into strands of a mesh depending on the skin (Fukunaga *et al.*, 2006). Deer have the least grouped fibrils and the greatest amount of single fibril fine mesh whereas pig has the most grouped fibrils and least single fibril mesh. It would seem that ovine material is extreme in that no grouped fibrils were observed at the surface, instead only the single fibril fine mesh observed.

The lack of grouped fibrils at the surface of ovine material is in contrast to AFM images generated from thin sections taken from the middle of both the enamel layer and the grain layer as illustrated in the figures below:

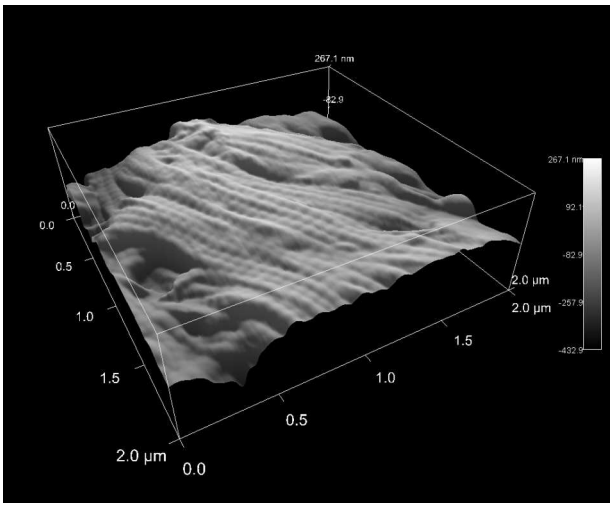


Figure 93: AFM image of the interior of the grain enamel of a conventionally processed pelt. Note the large grouping of fibrils not seen on the surface on the grain enamel.

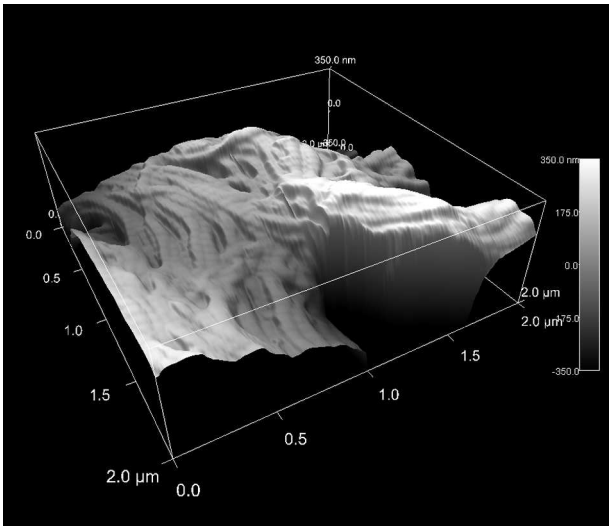


Figure 94: AFM image of the interior of the grain of a conventionally processed pelt. Again there is a large grouping of fibrils not seen on the surface on the grain enamel.

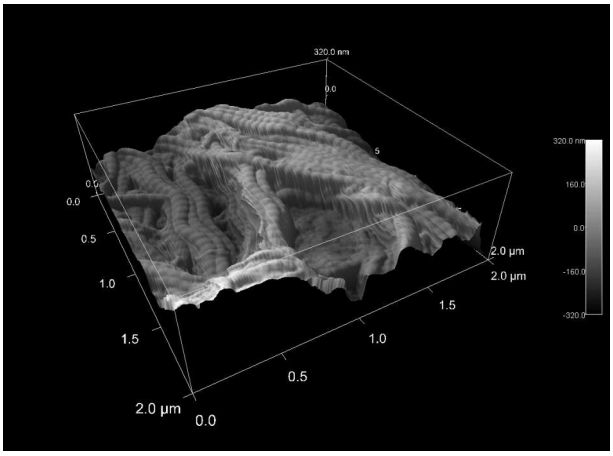


Figure 95: AFM image of the interior of the grain enamel of an enzymatically depilated pelt. Note the degree of disorganisation of fibrils in comparison to the conventionally processed material.

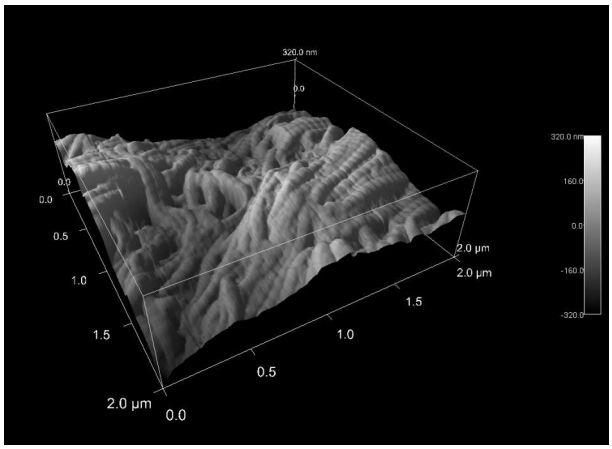


Figure 96: AFM image of the interior of the grain of an enzymatically depilated pelt. Note the degree of disorganisation of fibrils in comparison to the conventionally processed material.

It may be that the lack of grouped fibrils at the surface of ovine material in contrast to the internal structure leaves the collagen more susceptible to proteolytic damage during enzyme depilation and subsequent processing. The difference in surface topology noted between species may explain why it was indicated in the literature review that successful enzyme unhairing techniques may be being carried out non-ovine material but successful depilation of ovine material by enzymes without associated damage to the skin has not been achieved.

The finding that disruption of the fine mesh of individual collagen fibrils results from enzyme depilation is novel; The disruption of the fine mesh of individual collagen fibrils could allow easier access of reagents to the underlying strata in the skin, making the skin more susceptible to chemical damage, especially alkali damage because of the high pH of the solutions used. Such a change in structure at the molecular level could manifest itself as the weakening and dulling of the grain enamel so often observed at the macro scale after enzyme processing. If this is true, it follows that the event that causes the release of fibrils in the grain enamel is the underlying cause of the grain damage observed when skins are depilated using enzymes. Furthermore this suggestion is consistent with the earlier experiment investigating the nature of grain damage (4.2.2). Extreme levels of damage were observed on the grain when a skin had been exposed to enzymes and subsequently treated with an alkaline sulfide liming process. The earlier results point to the enzyme depilatory removing material from the surface structures

resulting in a new conformation that exposes disulfide cross links in the structural proteins which can then be cleaved by sulfide ions further enabling the alkaline dissolution of the structural proteins. This then explains both the extreme damage observed after subsequent sulfide treatment and damage observed to the surface after enzyme depilation.

It is possible that some sort of structure or molecule that binds the collagen fibril into a tightly woven mesh may be removed during processing. This finding also supports an earlier work which showed that enzyme treatment of pig skin caused a weakening of the affinity between fibrils which in turn lead to a separation of fibrils (Wang *et al.*, 2005)

5.5.2.2 2D SDS PAGE

In order to further investigate the grain damage caused by enzymatic depilation of skin, 2-D gel electrophoresis was carried out to analyse the proteins both in the enamel and within the remainder of the grain layer to identify any important differences in the protein complement of the different portions of skin using peptide mapping as illustrated in the figures below.

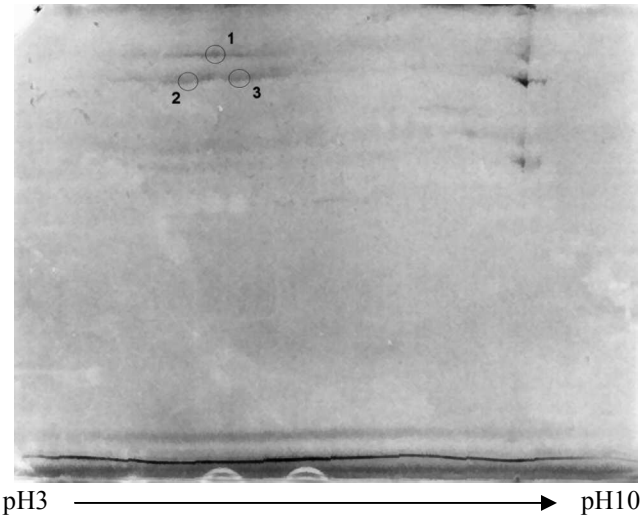
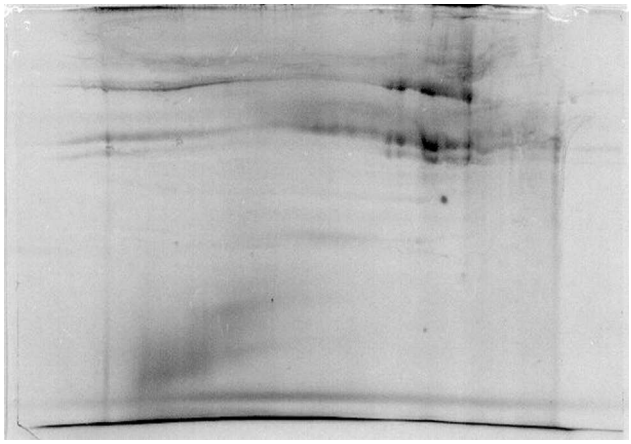
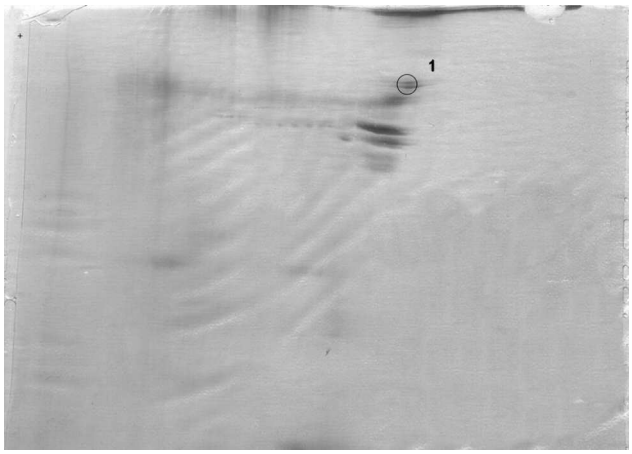


Figure 97: 2D electrophoretic (8 x 10cm) pattern of the sample sectioned from the enamel layer of the conventionally depilated skin. Note the spot labelled 1. Spot one was not identified in material sectioned from the remainder of the grain layer, indicating it is a protein that is relatively rich in the enamel layer. Spots two and three represent other proteins that are present in the enamel layer as well as in the remainder of the grain layers.



pH3 ⟶ pH10

Figure 98: 2D electrophoretic (8 x 10cm) pattern of the sample sectioned from the region of the grain underneath the enamel layer of the conventionally depilated skin. Note the absence of a spot in the location of spot 1 from Figure 97



pH4 ⟶ pH7

Figure 99: 2D electrophoretic (18 x 20cm) pattern of the sample sectioned from the enamel layer of the conventionally depilated skin. Note the spot labelled 1, which does not appear in other samples is split, possibly due to cleavage of the peptide or the partial removal of glycosaminoglycans. The entire spot was sampled for MALDI TOF analysis.

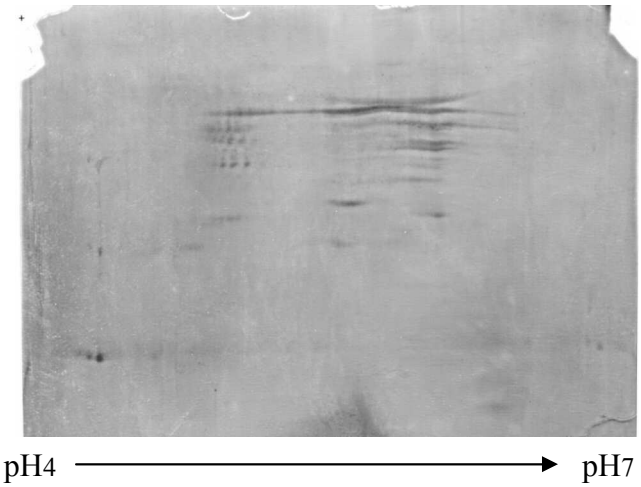


Figure 100: 2D electrophoretic (18 x 20cm) pattern of the sample sectioned from the enamel layer of the enzymatically depilated skin. Note the absence of a spot in the location of spot 1 from Figure 99. Also note the large number of proteins present in the enzyme depilated skin extract in comparison to the conventionally depilated skin.

The 2-D electrophoretic pattern of the enamel layer and the remainder of the grain layer exposed after removal of the enamel layer from both conventionally and enzymatically pickled skin extracts showed as expected that the majority of the proteins focussed between pH 5-6 (Deb Choudhury *et al.*, 2006).

Enrichment of the proteins in the enamel layer by selective microtome sectioning resulted in different electrophoretic patterns as can be seen in Figure 97 and Figure 98. The most abundant proteins in the enamel layer of conventionally depilated skin, spots 1, 2, and 3 (Figure 97) were analysed further. It is worth noting that spot 1 did not appear in the 2D gel of the section immediately below the enamel layer of the conventionally depilated skin (Figure 98), indicating that careful sectioning was able to differentiate the protein complement of the different layers of the skin.

5.5.2.3 MALDI TOF

In order to identify proteins lost or modified during enzymatic dewooling, spots 1, 2 and 3 (Figure 97) were excised from the 2-D gel of the conventionally processed enamel layer, and tryptic peptides generated from each spot. The samples were subjected to MALDI-TOF MS and proteins identified using the monoisotopic masses of the peaks generated from each spot (MASCOT, Matrix Science). The search parameters allowed for one missed cleavage, carbamidomethylation of cysteine residues and possible oxidation of methionine residues. The peptide tolerance for the experimental peptide

mass value was set at ± 1 Da and peptides from individual spots were matched with the monoisotopic masses of peptides from mammalian proteins. Sequences were also compared to those from bovine and human skin proteins as there is a shortage of protein sequence data from sheep proteins in the publicly accessible databases. Comparisons with bovine proteins have previously been reported on the assumption that only a few homologous peptide sequences are required for a successful match (Simpson *et al.*, 2004). Results are tabulated below:

Table 29: Proteins identified from spots in 2-D gel electrophoresis

Spot No.	Figure Table	Protein name	Source	UniProtKB/TrEMBL Entry name
1	Figure 97 Table 30	Collagen, type VI, alpha 2	Bos taurus	Q1JQB0_BOVIN
1	Figure 97 Table 31	Collagen, type VI, alpha 3, [Fragment]	Ovis aries	Q9MZW2_SHEEP
2	Figure 97 Table 32	Collagen, type III, alpha 1	Bos taurus	CO3A1_BOVIN[a]
1	Figure 99 Table 33	Collagen, type VI, alpha 2	Bos taurus	Q1JQB0_BOVIN
1	Figure 99 Table 34	Collagen, type VI, alpha 3, [Fragment]	Ovis aries	Q9MZW2_SHEEP
1	Figure 99 Table 35	Collagen, type VI, alpha 2 [Precursor - fragment]	Homo sapiens	CO6A2_HUMAN

[a] Originally referenced as PIR-PSD entry CGBO7S

Table 30: Observed and calculated masses of the tryptic peptides from spot 1 derived from Figure 97 as matched with UniProtKB/TrEMBL entry name Q1JQB0_BOVIN

Experimental m/z transformed to a relative molecular mass	Calculated relative molecular mass	Missed cleavage	Position of matched peptides
1180.593	1179.636	1	S^{120}-R^{129}
615.3927	615.334	0	L^{174}-R^{178}
602.2427	602.3024	0	E^{181}-R^{185}
587.2027	587.3391	1	G^{264}-K^{269}
1198.663	1199.524	0	G^{270}-K^{281}
807.3927	807.3511	0	G^{309}-R^{316}
669.3127	669.381	0	G^{318}-K^{325}
530.1927	529.3336	1	G^{333}-R^{337}

1328.663	1328.614	1	I^{338}-R^{350}
591.1327	590.266	0	G^{366}-K^{371}
825.1427	825.3981	0	G^{381}-K^{389}
796.3627	796.4191	1	G^{417}-R^{424}
574.2127	573.2758	0	G^{426}-K^{431}
1324.643	1325.621	1	G^{426}-K^{440}
1194.583	1194.563	1	G^{441}-R^{452}
1706.743	1705.838	1	G^{444}-K^{461}
923.4727	923.4937	1	G^{465}-R^{473}
840.4027	840.3726	0	G^{489}-R^{497}
841.4027	841.393	0	G^{498}-K^{506}
544.1927	543.2653	0	G^{521}-K^{526}
514.3127	514.25	0	G^{545}-R^{549}
572.2527	571.3693	0	L^{644}-K^{649}
988.5527	989.4778	1	D^{650}-R^{658}
896.4227	895.4651	0	I^{680}-K^{687}
745.3727	744.4494	1	L^{713}-R^{718}
1365.623	1364.643	1	H^{734}-R^{744}
1366.633	1366.68	0	N^{784}-K^{795}
1383.663	1382.675	0	N^{784}-K^{795}
1232.583	1232.567	0	T^{835}-R^{845}
1233.653	1232.713	0	L^{869}-R^{879}
524.1527	523.3231	0	L^{901}-R^{904}

Mass: 98215 **Score:** 213 **Expect:** 3.4e-16 **Queries matched:** 31 **Coverage:** 26%

Table 31: Observed and calculated masses of the tryptic peptides from spot 1 derived from Figure 97 as matched with UniProtKB/TrEMBL entry name Q9MZW2_SHEEP

Experimental m/z transformed to a relative molecular mass	Calculated relative molecular mass	Missed cleavage	Position of matched peptides
574.2127	573.3486	0	V^{1}-K^{6}
1717.793	1717.9	1	V^{25}-K^{40}
988.5527	989.4665	0	N^{32}-K^{40}
1037.523	1036.53	0	I^{41}-R^{50}
533.1627	532.2857	0	G^{109}-K^{113}
1302.663	1303.655	0	I^{127}-R^{138}

Mass: 27076 **Score:** 80 **Expect:** 0.0075 **Queries matched:** 6 **Coverage:** 19%

Table 32: Observed and calculated masses of the tryptic peptides from spot 2 derived from Figure 97 as matched with UniProtKB/TrEMBL entry name CO3A1_BOVIN

Experimental m/z transformed to a relative molecular mass	Calculated relative molecular mass	Missed cleavage	Position of matched peptides
1648.851	1648.851	1	G^{153}-R^{170}
2054.029	2054.029	1	G^{258}-K^{280}
1707.781	1707.781	0	D^{311}-R^{329}
631.3441	631.3442	0	G^{330}-R^{335}
764.3929	764.3929	0	G^{354}-R^{362}
1320.679	1320.679	0	G^{624}-R^{638}
2719.831	2719.331	1	G^{672}-K^{704}
839.4501	839.4501	0	G^{813}-K^{821}
948.5028	948.5029	0	G^{984}-K^{994}

Mass: 93708 **Score:** 78 **Expect:** 0.01 **Queries matched:** 9 **Coverage:** 13%

Table 33: Observed and calculated masses of the tryptic peptides from spot 1 derived from Figure 99 as matched with UniProtKB/TrEMBL entry name Q1JQB0_BOVIN

Experimental m/z transformed to a relative molecular mass	Calculated relative molecular mass	Missed cleavage	Position of matched peptides
2705.159	2704.412	1	H^{147}-K^{173}
614.3877	615.334	0	L^{174}-R^{178}
1350.663	1349.701	0	V^{248}-K^{260}
2150.967	2150.116	1	Q^{284}-K^{305}
806.4067	807.3511	0	G^{309}-R^{316}
1054.517	1053.575	1	L^{335}-K^{344}
981.4637	981.4992	1	R^{380}-K^{389}
1193.585	1194.563	1	G^{441}-R^{452}
840.3997	840.3726	0	G^{489}-R^{497}
543.1727	543.2653	0	G^{521}-K^{526}
514.3157	514.25	0	G^{545}-R^{549}
988.5607	989.4778	1	D^{650}-R^{658}
896.4307	895.4651	0	I^{680}-K^{687}
2367.192	2366.206	1	N^{692}-K^{712}
744.3877	744.4494	1	L^{713}-R^{718}
1364.633	1364.643	1	H^{734}-R^{744}
1233.656	1232.713	0	L^{869}-R^{879}
2064.035	2063.03	1	Q^{889}-R^{904}

Mass: 98215 **Score:** 133 **Expect:** 3.4e-08 **Queries matched:** 17 **Coverage:** 21%

Table 34: Observed and calculated masses of the tryptic peptides from spot 1 derived from Figure 99 as matched with UniProtKB/TrEMBL entry name Q9MZW2_SHEEP

Experimental m/z transformed to a relative molecular mass	Calculated relative molecular mass	Missed cleavage	Position of matched peptides
988.56	989.466	0	N^{32}-K^{40}
1035.532	1036.53	0	I^{41}-R^{50}
1649.769	1650.751	0	A^{7}-R^{21}
2007.896	2007.985	1	N^{32}-R^{50}
2565.162	2565.263	1	A^{86}-K^{108}

Mass: 27076 **Score:** 72 **Expect:** 0.047 **Queries matched:** 5 **Coverage:** 23%

Table 35: Observed and calculated masses of the tryptic peptides from spot 1 derived from Figure 99 as matched with UniProtKB/TrEMBL entry name CO6A2_HUMAN

Experimental m/z transformed to a relative molecular mass	Calculated relative molecular mass	Missed cleavage	Position of matched peptides
1340.0117	1339.681	0	V^{4}-K^{16}
1715.8157	1715.867	1	V^{4}-R^{19}
2150.9667	2150.116	1	Q^{40}-K^{61}
806.4067	807.3511	0	G^{65}-R^{72}
1054.5167	1053.575	1	L^{91}-K^{100}
614.3877	614.2772	0	G^{101}-R^{106}
2039.9527	2040.889	1	G^{101}-R^{121}
1987.8557	1986.867	1	G^{107}-K^{127}
981.4637	980.5403	1	R^{136}-K^{145}
824.1607	824.4392	0	G^{137}-K^{145}
1193.5847	1194.563	1	G^{197}-R^{208}
1587.6947	1586.853	1	G^{224}-K^{240}
1437.7197	1437.732	1	G^{230}-R^{244}
840.3997	840.3726	0	G^{245}-R^{253}
1164.5827	1164.564	1	G^{283}-R^{294}

Mass: 33294 **Score:** 183 **Expect:** 3.4e-13 **Queries matched:** 15 **Coverage:** 42%

Spot 1, which was absent from the 2D-gel profiles generated from the grain layer of the conventionally treated skins, showed good correlation with peptides from collagen type

VI of both bovine and ovine skin (Table 29, Table 30, and Table 31), indicating significant homology between collagens from these two species supporting the assertion that the antibodies to collagen VI used in sections 5.4.3.2 and 5.4.4.3 were reactive to ovine collagen VI. Spots 2 and 3 appeared in the gels of both the enamel and grain layers, (Figure 97 and Figure 98 respectively) with spot 2 identified as type III collagen (Table 29 and Table 32).

In order to confirm these results, and more specifically the presence or absence of collagen VI in the enamel layers of conventionally and enzymatically depilated skins, large format 2D gel electrophoresis was carried out over a narrower pH range (pH 4-7) (Figure 99 and Figure 100). The major difference between the 2D patterns of the enamel layers was the absence of a significant number of proteins from the conventionally depilated material that were present in the enzymatically depilated material. An exception to this trend was the absence in Figure 100 of spot 1 in Figure 99, which was subsequently identified as collagen VI (Table 33, Table 34, and Table 35) confirming the results obtained for the small format gels. This difference validates important implications noted earlier for explaining the effects on the enamel integrity of enzyme depilated skins.

The fibre-forming collagens present in skin are mainly types I and III, and, to a lesser extent, type V. Any of these types can form fibrils in combination with each other or on their own. Type I and III collagens in particular, have been shown to resist liming, bating and pickling (Kronick & Iandola, 1997); (Kronick & Iandola, 1998).
Collagen VI, however, has been shown to react differently to proteolytic attack. Collagen VI has been shown to be resistant to collagenase under non-reducing conditions in contrast to collagens I – V indicating that there are differences in its structure (Heller-Harrison & Carter, 1984). It is, therefore, not surprising that it confers specific properties to the tissue in which it is found.

Previous immunolocalization studies showed that collagen VI microfibrils form extensive filamentous networks intercalated between interstitial collagen I and II fibrils (Kuo *et al.*, 1997); (Cay *et al.*, 1997); (Keene *et al.*, 1998) where they act to anchor them to the basement membranes (Zeichen *et al.*, 1999); (Keene *et al.*, 1988). Collagen VI monomers consist of a short triple helical domain with a large globular domain at

each end (Chu *et al.*, 1990), and have been shown to assemble intra-cellularly into dimers and tetramers (Engvall *et al.*, 1986). These are then secreted into the extracellular matrix where they assemble into microfibrillar structures by end-to-end association (Engvall *et al.*, 1986). The structural features of collagen VI, along with its Arg-Gly-Asp cell binding sequences in the triple helical domain, make it ideally suited to playing a role in cell-matrix and matrix-matrix association (Aumailley *et al.*, 1991). Furthermore, "Von Willebrand factor A" domains (specific collagen binding domains) in collagen VI are not only thought to be involved in self interactions but also appear to be involved in interactions with fibrillar proteins such as collagen type I (Veit *et al.*, 2006a)

Evidence for the presence of collagen VI in the grain enamel layer which persists even after conventional processing up to the pickled pelt stage, was reported earlier (Kronick *et al.*, 1991) and is supported by the immunolocalization studies earlier on in this work. The presence of non-fibrillar collagens such as collagen VI have been shown to be concentrated on the surface of the fibrils (Keene *et al.*, 1988) probably enhancing the ability of the fibres and fibrils to be very tightly associated with each other (Kronick & Iandola, 1997).

5.5.3 Stratified samples – Summary

This work confirms other reports positioning collagen VI in the grain enamel and also shows that collagen VI is removed from the surface collagen fibrils during enzymatic depilation. Due to its association with collagen type I fibrils and its "adhesive" function in protein-protein interactions, the removal of collagen VI might result in the loosening of the fibril mesh at the skin surface as observed (Figure 91 and Figure 92). It is therefore possible that the removal of collagen VI, through enzymatic depilation could cause a reduction in the stability of the fibrillar mesh structure at the skin surface.

Removal of collagen VI from the enamel layer of the skin is consistent with a deterioration of the fine structure at the skin surface and may be the underlying cause of the damage inevitably observed on the surface of enzymatically depilated skins. The release of collagen type I fibrils from the surface in the form of a loosening of their structure via destruction of collagen VI is a novel concept. This possibility explains why

skins can appear damaged after enzyme treatment even though the main component of the skin surface is collagen I which is relatively stable to enzyme hydrolysis. This knowledge will assist in the development of better enzymatic processing regimes for the leather industry.

5.6 Determination of the nature of changes in the proteins – Conclusions

Two approaches to determine the effects of enzyme depilation on skin protein at the molecular level were described in this chapter. The first investigation determined what proteins need to be removed or destroyed during enzyme depilation to achieve efficient depilation. The second determined which proteins remain in successfully depilated and undamaged skin. The following conclusions were made from the results.

An investigation into the mechanism of enzymatic depilation using a range of proteolytic enzymes showed that destruction of the basal layer of the skin with the concomitant removal or destruction of some of the cells of the root sheaths is accompanied by loosening of the wool fibre in the root allowing depilation.

The removal of proteoglycans and or the hydrolysis of glycosaminoglycans from glycoproteins appears to be positively correlated with efficient depilation indicating that the inclusion of a glycosidase in an enzymatic depilation process is warranted.

Elastin may be important in the grain surface and was removed from the grain during enzymatic depilation indicating that its removal during enzymatic depilation may be related to looseness in finished leather that has been observed after enzyme processing.

Collagen VI appears to be destroyed by enzymatic depilation, and its location in the grain layer strongly suggests that its destruction is correlated to the release of fine collagen fibres from the enamel resulting in a poor quality product.

The key to successful enzymatic depilation appears to be the destruction of as many different proteins as possible with the absolute exception of the minor fibrous collagens including collagen VI in particular.

Controlling undesirable enzyme activity

It was shown in chapter 5 that the key to successful enzymatic depilation is the retention of certain minor proteins. The objectives of this chapter therefore were to investigate means to achieve an improved selectivity for the retention of those proteins that appear to maintain a high quality surface. The options approached for modifying the depilation process were two fold:

Firstly modification of the specificity of a selection of enzymes known to depilate. For example modification of the activity of the enzymes through the use of additives such as sodium chloride (Na Cl). A number of enzymes were investigated in chapter 3 which had depilatory activity. Each one depilated to a different extent and each caused some level of damage. They therefore represent a good set of enzymes to use to examine possible modifications of the depilation system in order to improve depilation and reduce damage.

Secondly selection of a new enzyme that has a good activity against proteins of the extracellular matrix such as laminin but reduced activity against minor fibrous collagens. The difficulties with this approach are that locating an enzyme with the opposing attributes of broad spectrum general activity with limited activity against all collagens may be difficult, and that it is likely that such an enzyme if located may be available in quantities too small to carry out simple depilation experiments. As such the development of a method for examining the depilation and damaging potential of micro-quantities of enzyme would be required

6.1 Salt addition

The addition of salts to enzymes can selectively alter their biochemical properties, including their activity against different substrates (Baldwin, 1996); (Mozersky *et al.*, 2005). An experiment was therefore carried out to investigate the impact of salts on the activity of the range of proteolytic enzymes initially examined.

6.1.1 Determination of activity against general skin substance

In order to evaluate the impact of salt on the activity of the enzymes investigated an assay was required. An assay for the activity against the proteins in ovine skin was developed in chapter 3. The effect of salt on enzyme activity was therefore examined using this skin powder azure (SPA) technique as described in section 3.3.1.2.

6.1.1.1 Method

100mg of skin powder azure was weighed into a 50mL conical flask. Fifteen mL of buffered sodium chloride solution was added (0, 5, 10, 15, 20 %w/v NaCl in 50 mM $NaHCO_3$ at pH 10.5) and the conical flasks were placed in a 35°C shaking water bath.

Sequential 1mL samples were taken every 10 seconds after the addition of the enzyme and discharged directly into 5 ML of a 5% (w/v) trichloroacetic acid solution to quench the reaction.

Samples were centrifuged at 2000g for 30 minutes and absorbances of the supernatants were measured at 595nm. The initial rate of change of absorbance per unit time was then determined. The effect of sodium chloride on general skin proteolytic activity for each of the enzymes examined are shown in Figure 101

All enzymes showed a general reduction in activity against skin substance with increasing salt concentration. In order to examine whether this modification of enzyme activity would protect the grain surface while allowing depilation to continue, sodium chloride concentrations that achieved a 20% reduction in enzyme activity were trialled. A 20% level of reduction was chosen because it was achievable for all the enzymes tested, while also providing residual activities that would allow potentially feasible industrial scale depilation The test was carried out to see if this level of reduction in activity would result in an observable improvement in the quality of enzyme depilated skins.

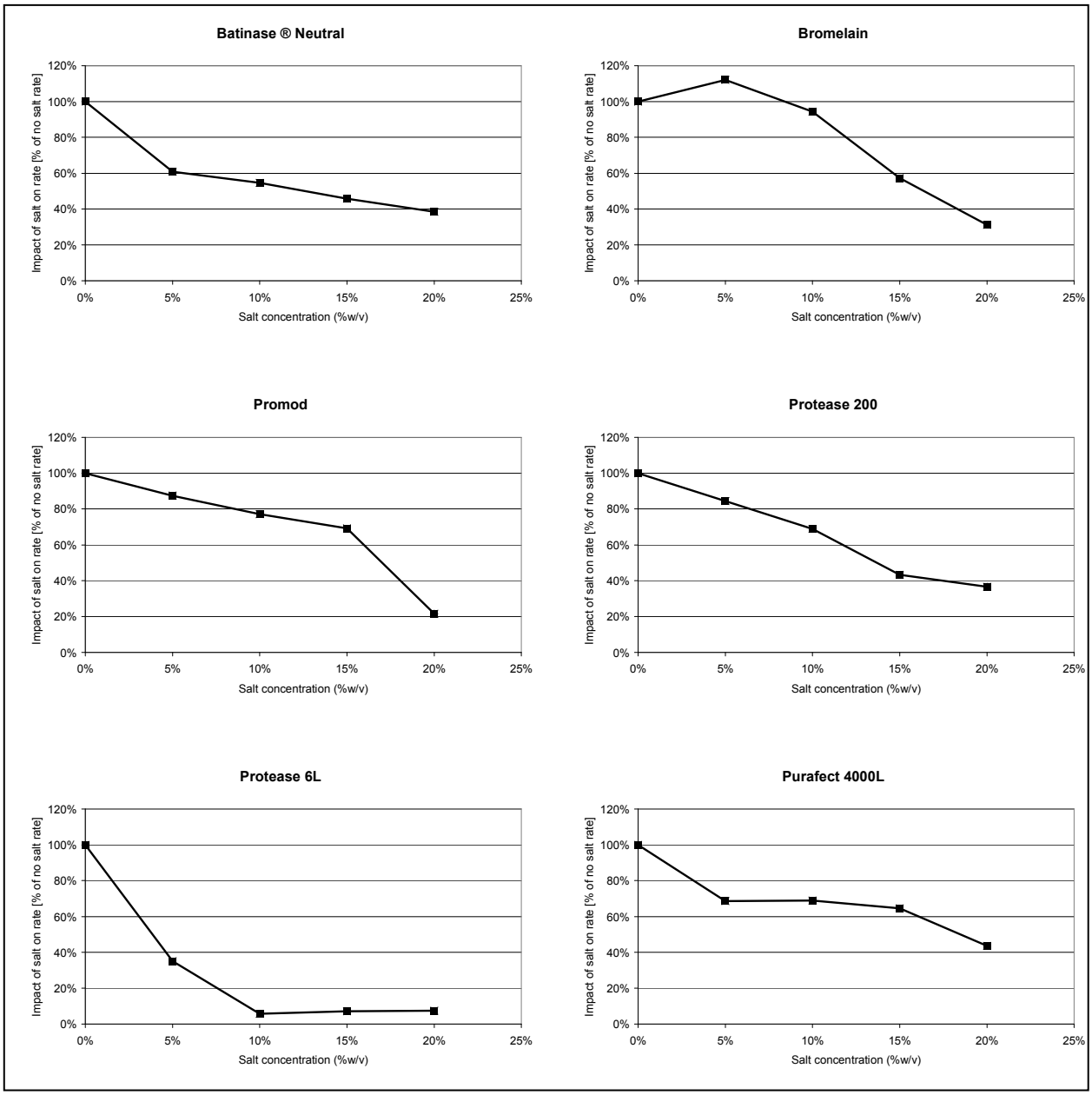


Figure 101: Initial experiment for enzyme activity vs. Sodium chloride concentration response. The error for this test was previously determined to be 0.0018 AU/s or about 20% of the measured rate (section 3.3.1.4). Note the general reduction in activity with respect to the addition of sodium chloride.

6.1.2 Salt depilation method

The enzyme formulation used in the depilation trials was the same as in section 3.2.1 except for the addition of sodium chloride prior to making up to final volume. The final NaCl concentration calculated to give a 20% reduction in activity against the skin substance based in enzyme assays (section 6.1.1) is shown in Table 36.

Table 36: Final NaCl concentrations used with the different enzyme formulations

Name	% Salt (wt/vol)
Batinase ® Neutral	2%
Bromelain	10%
Promod 278P	9%
Protease 200	6%
Protease 6L	3%
Purafect 4000L	3%

Pieces of raw skin (5×5cm) (from the OSP region(IUP 2, 2000)) were soaked fully immersed in enzyme solution for 60 minutes. After this wet holding period, the pieces were pressed to recover the residual free liquor and then held overnight (16 hrs) at 20°C. They were assessed for the ease of depilation and the quantity of residual wool was estimated. Samples were also taken to assess the impact on grain structures during depilation. The pieces were then pickled immediately in the LASRA standard pickle (appendix 8.2.3) and processed to dyed crust leather using the standard LASRA method (appendices 8.2.5, 8.2.6). The grain surface of the crust leather samples were then examined under a binocular microscope at 5× magnification.

6.1.3 Depilation results

Table 37: Enzyme effect on ease of depilation and wool removal

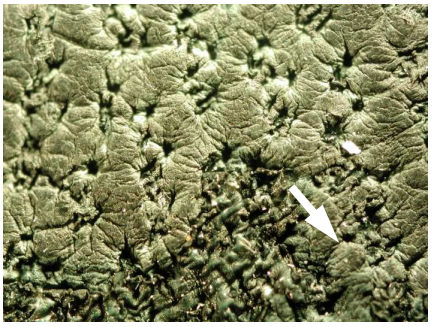
Name	Ease of depilation	% Wool removed
Batinase ® Neutral	Very easy	100%
Bromelain	Easy	99%
Promod 278P	Easy	98%
Protease 200	Difficult	80%
Protease 6L	Easy	100%
Purafect 4000L	Very easy	100%

In most tests high levels of depilation were maintained with added salt, the exception being Protease 200 which showed a marked reduction in the ease of depilation after the addition of salt. The results showed that the addition of sodium chloride at a concentration that achieved a 20% reduction in activity against skin proteins did not excessively inhibit depilation. The grain surfaces of the tanned skin samples were examined under the light microscope at 5× magnification. Typical examples are illustrated in Figure 102.

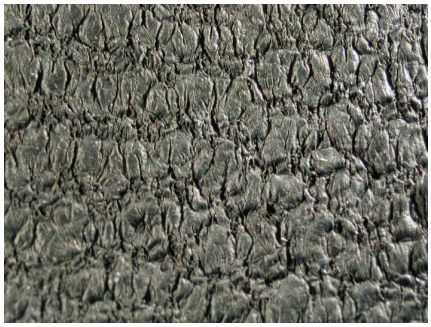
Batinase ® Neutral

Bromelain

Promod 278P

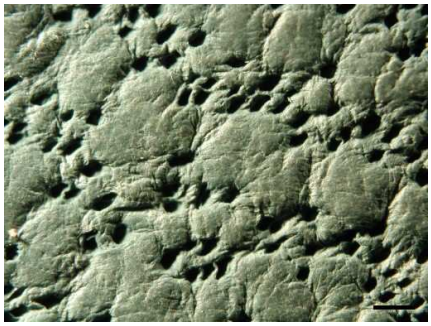


Protease 200



Protease 6L

Purafect 4000L



Standard conventionally depilated

Figure 102: Effect of sodium chloride on grain surface examined at 5× magnification (arrows indicate regions described in Table 38).

Table 38: Effect of sodium chloride on grain surface

Name	Follicular pads	Between pads	Other comments
Promod 278P	Bright	Light surface breakdown	Heavily drawn grain surface
Protease 6L	Bright	Medium surface breakdown	Drawn grain surface
Protease 200	Bright	Bright, irregular dying	Regions of epidermis remain (arrowed)
Batinase ® Neutral	Bright	Bright	Large cracks revealing fibrous substrata (arrowed)
Bromelain	Bright	Bright	Isolated cracks revealing fibrous substrata (arrowed)
Purafect 4000L	Bright	Isolated light surface breakdown	-

For all enzymes there was an improvement in the skin surface appearance compared to skin depilated in the absence of sodium chloride (Table 8). The Protease 200 treated skin also exhibited a marked improvement in the visual appearance of the grain surface. However, as was noted above, the efficiency of depilation was significantly attenuated for this enzyme. It is therefore likely that if the skin had been exposed for longer using these conditions to achieve complete depilation, damage to the skin surface would have also occurred.

For all enzymes the inter-follicular pads were flattened in comparison to traditionally sulfide depilated skins. The effect is analogous to an air filled balloon which has leaked air. Some skins showed more severe effects than others. The enzymes are ranked in Table 39 according to the appearance of the inter-follicular pads.

Table 39: Grain flattening effect

Name	Follicular pads
Purafect 4000L	Most flattened
Promod 278P	↓
Batinase ® Neutral	
Protease 6L	
Bromelain	
Protease 200	Most full

Comparison of the leather produced with and without added sodium chloride, showed that overall, the flattening effect was reduced by the addition of sodium chloride. Although Protease 200 resulted in the least flattened effect, the reduction in damaging activity through the use of salt also seemed to have a negative impact on the efficacy of depilation.

In order to determine how the addition of sodium chloride altered the depilation process, samples were taken and the effect of salted depilation was assessed using the SACPIC staining technique.

6.1.4 The effect of sodium chloride on grain structure

The SACPIC follicle staining technique was used to assess the impact of salt addition on the grain structures during enzyme depilation. The pelt, the outer root sheath (ORS), the epidermis, and the wool root bulb were all examined using the methods described in section 8.3.3 with the results summarised in Table 40.

Table 40: Effect of enzyme depilation in the presence of salt on grain microstructures

Name	Epidermis	Grain	Upper ORS	Lower ORS	Bulb
Batinase ® Neutral	All removed.	Mostly intact; small amount of damage observed in follicle mouths.	Only small quantities of debris remain.	Only small quantities of debris remain.	Only small quantities of debris remain.
Bromelain	All removed.	Scuffing over most of grain surface; Heaviest at follicle mouths. Damage penetrates into grain.	Gone from surface; Some portions remain inside the grain with debris.	Mostly intact; Some debris within.	Mostly intact; Some debris within.
Promod	Some particles remain in follicle mouths.	Mostly intact; Small regions of damage observed all over grain surface.	Mostly intact; Some debris within.	Only small quantities of debris remain.	Mostly intact; some debris within.
Protease 200	All removed	Scuffing over most of grain surface; Heaviest at follicle mouths.	Gone from surface; Some portions remain inside the grain with debris.	Mostly intact; Some debris within.	Mostly intact; Some debris within.
Protease 6L	All removed	Mostly intact. Small amount of damage observed in follicle mouths	Only small quantities of debris remain.	Only small quantities of debris remain.	Only small quantities of debris remain.
Purafect 4000L	All removed	Scuffing over most of grain surface.	Gone from surface; Some portions remain inside the grain with debris.	Some still intact; Some debris within.	Some still intact; some debris within.

It has previously been noted that a successful depilation enzyme should break down the cells of the ORS enabling easy removal of the wool fibre whilst leaving the collagen and grain surface intact. Each enzyme showed a reduced level of damage to the grain surface in the presence of sodium chloride with respect to the equivalent non salted depilated sections (section 5.3.2). In particular, some examples showed there was less damage to the inter-follicular pad surfaces. Unfortunately however, the sodium chloride did not provide complete protection to the grain surface of all the samples tested. Even

skin depilated with Protease 200, which appeared to be undamaged using surface light microscopy, had observable damage to the grain surface and at the follicle mouths when sections treated with the SACPIC stain were viewed. Residual damage within the follicle mouths indicated that there was some unwanted activity in the process.

Overall, the addition of sodium chloride to the enzyme mix resulted in a reduction of undesirable activity under the assay condition and resulted in improvement in grain quality. While high levels of depilation were maintained, the addition of sodium chloride did not completely prevent surface damage or inter-follicular pad flattening. It is possible that other salts might provide improved protection to the grain surface without compromising depilation.

6.1.5 Alternative salts

It is possible that alternative salts might have different effects on the depilation process because of their impact on the stability of the various proteins present (Baldwin, 1996) as well as the activity of the enzymes in the process. The position of the cations and anions making up the salt in the lyotropic series will affect its properties and hence its interaction with various proteins. Salts may be considered either lyotropic (complex forming) or chaotrophic (complex breaking) and thus influence the way in which proteins interact with one another. This can and does have an affect on substrate enzyme interactions.

6.1.6 Method

Different salts were added to the depilation solution (pH of 10.5) at a range of concentrations. Maintaining the pH in the presence of the different salts was important to ensure that any effect observed was related to the salt itself and not a change in the pH. The high pH restricted the possible cations to sodium, lithium, and potassium. Salts of each of these metals were chosen such that they were soluble to a concentration of at least 20% w/v in order to examine a large concentration range. As lithium carbonate is relatively insoluble a 50mM glycine buffer was used for each of these experiments instead of the carbonate buffer previously described in section 6.1.1

6.1.7 Salt solution formulation

100mL of the salt solutions were made up using the following formulation: x% salt, where x = 0%, 5%, 10%, 15%, and 20% w/v, where the salts were: sodium chloride, sodium sulfate, lithium chloride, lithium sulfate, potassium sulfate, or potassium carbonate. All salts were prepared in a 50mM glycine buffer.

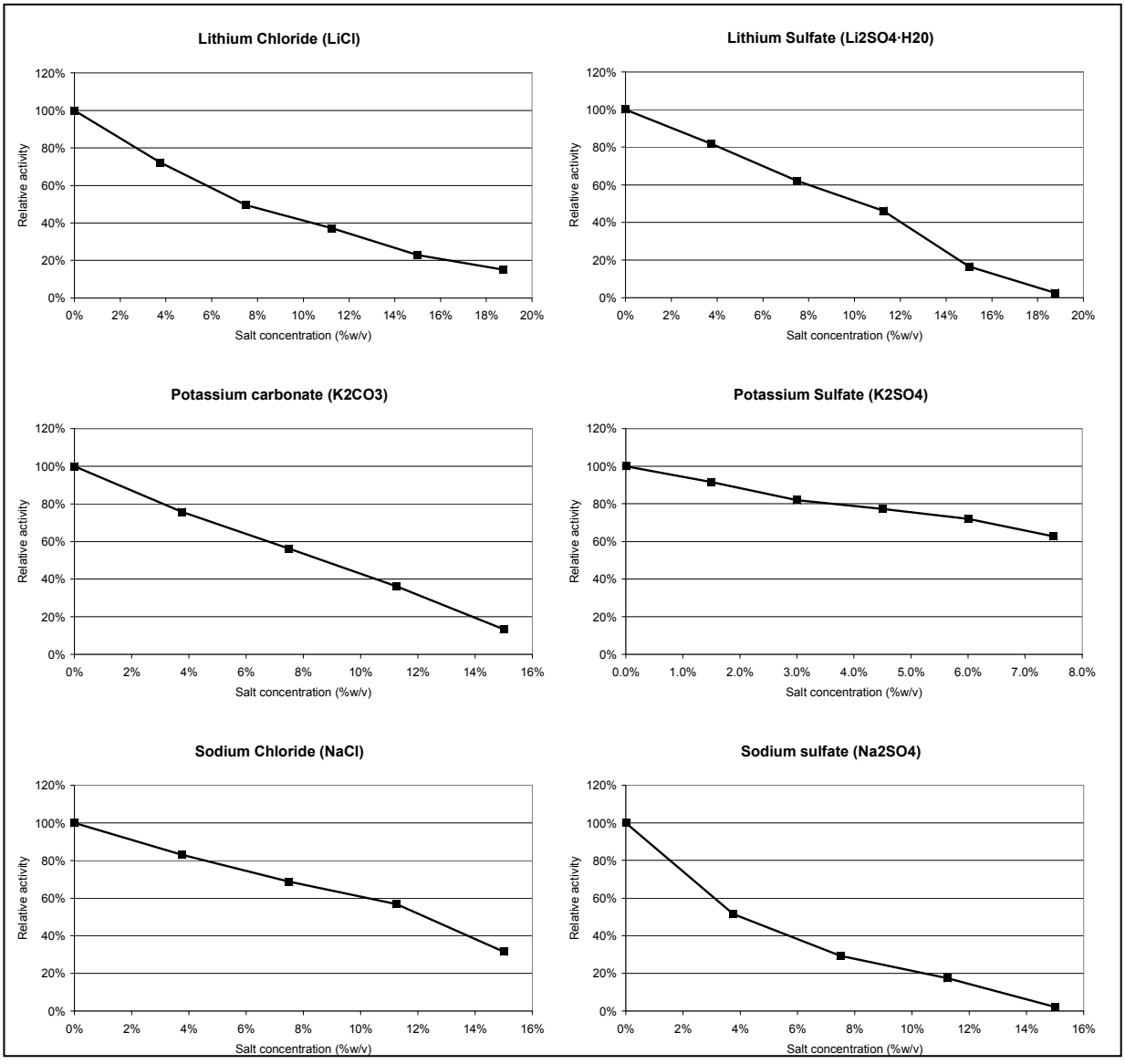


Figure 103: Impact of various salts on enzyme activity against general skin substance

Each salt solution was then adjusted to pH 10.5 using 10%w/v aqueous solutions of the hydroxide of each respective salt cation. A buffered enzyme solution of each salt was prepared using the most successful enzyme in terms of depilation efficiency and level of grain damage (Purafect 4000L) and the assay was then carried out as described in

section 6.1.1. The results are displayed as a plot of enzyme activity as measured by the method described in section 3.3.1.2. verses the concentration of the salt used.

The results for each of the salts examined are plotted in Figure 103 with salt concentration on the x axis and relative reaction rate on the y axis. The results show that increasing concentrations of all salts caused a reduction in enzyme activity against skin substance. In order to examine whether any given salt would protect the grain surface while allowing depilation to continue the salt concentrations required to achieve a 20% reduction in activity were used in an enzyme depilation study, as performed above with sodium chloride. A 20% level of reduction was chosen because it could be achieved for all the salts tested and would be expected to have an observable effect on depilation under laboratory conditions.

6.1.8 Batch trial methodology

Skins samples from the OSP region of the lamb skin were taken and processed as described above in section 3.2.1 except pre-soaking was performed in the presence of the prepared salt solutions. The concentrations of the salts used to achieve a 20% reduction in enzyme activity are given in Table 41.

Table 41: Salt concentrations imparting 20% reduction in activity against skin powder azure (SPA)

Salt	% w/v applied in batch
Lithium chloride	2.7%
Lithium sulfate monohydrate	4.1%
Potassium carbonate	3.3%
Potassium sulfate	3.8%
Sodium chloride	4.0%
Sodium sulfate	1.5%

Enzyme depilation was then carried out using the procedure described in section 6.1.2

6.1.9 Results

The depilation results are given in Table 42.

Table 42: Impact of various salts on depilation

Salt	% depilation
Lithium chloride	99%
Lithium sulfate	100%
Potassium carbonate	80%
Potassium sulfate	5%
Sodium chloride	70%
Sodium sulfate	20%

Placing the salts in order of depilation efficiency for a 20% reduction in enzyme activity gives Li_2SO_4>LiCl>K_2CO_3>NaCl>Na_2SO_4>K_2SO_4. Since the salts were added to a concentration that gave a 20% reduction in activity against general skin substance, it was encouraging to see 99 and 100% depilation in the presence of lithium salts. This indicated that depilation efficiency was not lost by reducing the activity of the enzymes on skin substance by addition of these salts. The presence of potassium sulfate resulted in such poor depilation, that the skin piece was held under the same conditions for a further 48 hours at which time it was 99% depilated. The pieces were then processed to crust and examined for the effect of salt addition on the grain surface. The results are illustrated in Figure 104.

It was evident that the addition of lithium sulfate to the enzyme depilation mix resulted in a grain surface that was most like conventionally sulfide depilated skins. It, however, was still not as good as conventionally depilated material with damage to the follicle mouths visible.

Potassium sulfate also appeared to result in a good grain surface but took a much longer time for acceptable levels of depilation (64 hours). It would be an interesting exercise to investigate the effects of potassium sulfate on enzyme depilation at the molecular level since the damage was so reduced. If the effect of this salt could be replicated over a much shorter time span, and evenly over a whole skin it may be a promising route to successful enzymatic depilation. It is possible however that by reducing the overall activity, the width of the depilated and un damaged area (as described in chapter 4) has become so large as to allow depilation without time for the damage to occur. As such

the general mechanism of depilation and damage may remain essentially unchanged. If this is the case then speeding the process of depilation up in the presence of potassium sulfate is unlikely to result in an un-damaged and depilated pelt.

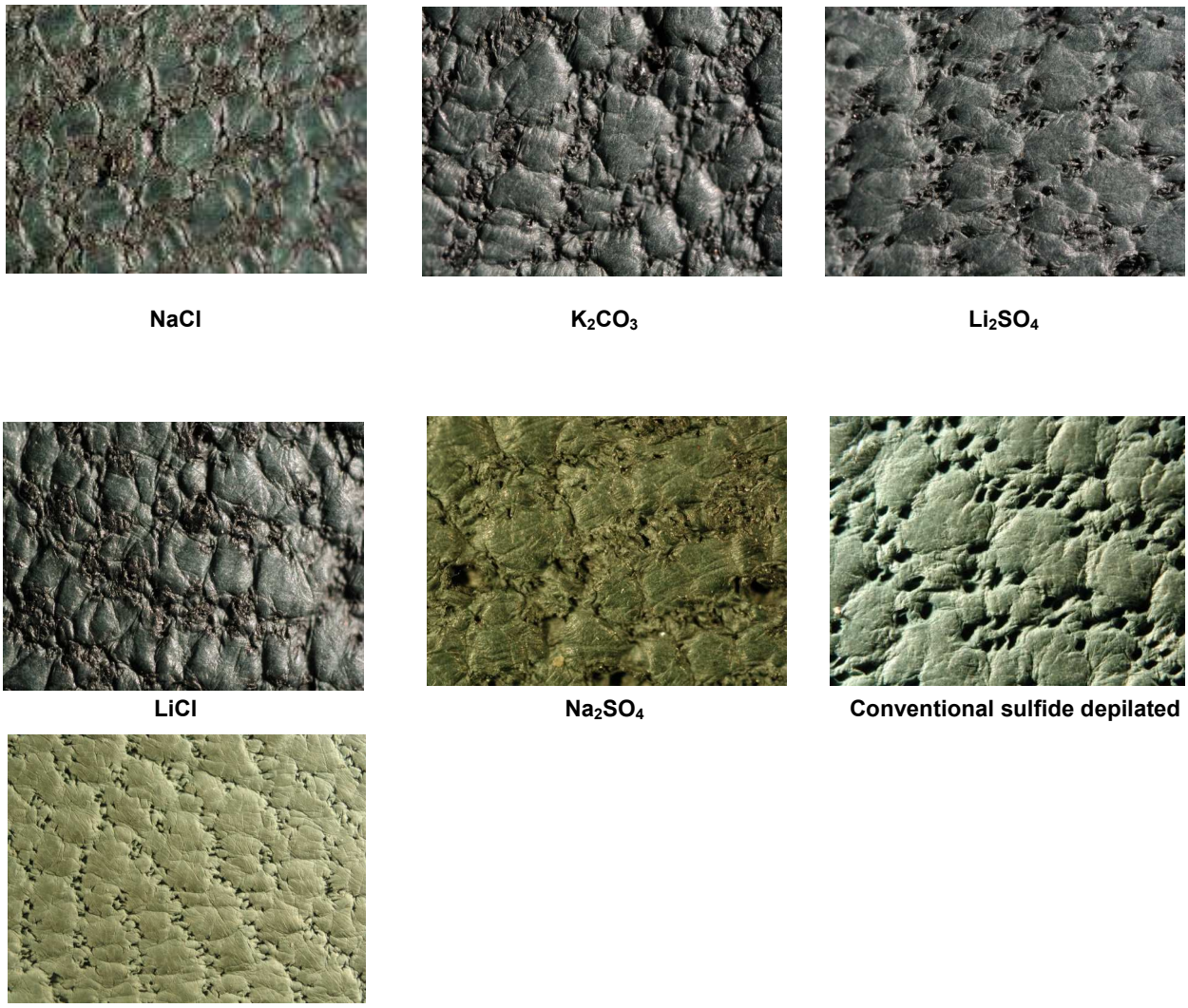


Figure 104: Impact of various salts on grain surfaces

Furthermore, although the small sample from the OSP used in this experiment was cleanly depilated with no damage after the extended reduced activity depilation, this does not imply necessarily that the whole skin would be depilated without damage. Particularly because it was found in chapter 4 that there was a high level of variability in enzymatic depilation in comparison to size of the region of skin which is not damaged.

6.1.10 Addition of salt – Summary

The investigations showed that the addition of salt to the enzyme depilation mixture had a positive effect on controlling damage to the proteins of the grain. While potassium sulfate generated a depilated skin with a good quality surface, the depilatory activity was reduced to the point that the process took three days. It is possible that the three day process was not a result of the enzyme activity but was due to the salt itself or the results of microbial contamination, or endogenous enzymes released over the period (see section 2.4.10.3). While the surface of the skin indicated the possibility of being able to produce high quality lamb pelts, the time necessary for complete depilation was unsuitable for industry and was therefore not further investigated.

Overall the addition of salt may be a means to modulate the damaging activity of enzymatic depilation. It is possible that adding salt may reduce the enzyme activity but leave the rate of diffusion relatively unchanged. Changing the relative activity of the enzyme in comparison to its rate of diffusion may lead to a process whereby the enzyme can penetrate the skin more evenly to the appropriate regions of the wool root sheath and bulb resulting in a more even depilation and hence better quality product with less damage.

6.2 Enzyme Specificity

Work in chapter 5 showed that certain proteins are important in maintaining good quality leather after enzymatic depilation. Work in section 6.1 showed that the activities of some depilation enzymes against general skin substance could be reduced by the addition of specific salts. This is supported by earlier work (Cantera, 2003) where it was shown that the relative ability of enzymes to hydrolyse different skin substrates can be modified through the use of salt. Furthermore the effect of enzyme activity on the grain surface may have also been altered relative to its ability to depilate.

It was noted in section 2.8 that proteolytic enzymes exist which differentially hydrolyse specific proteins in the extracellular matrix. In this work it is postulated that successful enzymatic depilation could be achieved by using an ECM protease that does not degrade collagen VI. Many proteases described in Table 4 degrade collagen which is

detrimental to the depilation process because, as noted earlier, the minor protein collagen VI may have a major role in maintaining the integrity of the surface collagen fibrillar network. This work has shown that the non collagenous proteins that surround the cells of the root sheath are a better target for successful depilation. Therefore a protease with no collagenase activity such as MMP-11 or Dispase might prove to be an option for depilation. A number of pure enzymes were therefore trialled to test this hypothesis.

6.2.1 Single section depilation method SACPIC visualisation

Due to the limited availability of pure enzymes which are reported to have no activity against collagen VI, a method had to be developed to determine their depilation effectiveness on a microscopic scale. Previous work with small quantities of enzyme (as low as 20 ug) have been developed that involve injection of enzyme into the skin (Yates, 1969b). A method was developed with the SACPIC stain described earlier that allowed changes in the surface proteins and structures involved with depilation to be visualised directly. Enzyme of a suitable concentration was applied to a single 20 um thick section of lambskin on a microscope slide for 16 hours at 20°C. The section was then stained with the SACPIC stain (appendix 8.3.3).

To assess the feasibility of this method trials were carried out using Purafect 4000L (a known depilatory) and the results compared to sections taken from successfully enzymatically depilated samples prepared earlier (section 5.4.2).

Sections for depilation were prepared by taking samples of frozen raw skin 10mm long by 3mm wide, cutting 20 um thick sections at right angles to the skin direction, and air fixing them on a glass microscope slide. The sections were then held in a sealed container at -20°C until required.

In the initial experiments using Purafect (section 3.2), 2% w/w enzyme in a carbonate buffer system was used. It was noted in the immuno-histochemistry experiments that 20 uL of solution completely wetted each skin section. Assuming a density of about 1000 $kg.m^{-3}$ for the skin sections used, this corresponds to a skin weight of 600 ug which would require the addition of about 0.6 uL of a 2% enzyme solution to be equivalent to

the amount of enzyme used in previous experiments. The requirement of such low amounts of enzyme was ideal for investigating more specific enzymes. As 0.6 uL of solution was unlikely to keep the section wetted for 16 hours it was necessary to modify the method. A series of development experiments were therefore carried out to refine the proposed technique so that it could be used routinely.

6.2.2 Single section depilation method development

The first step was to determine the required enzyme concentration and application method

A single cryosection was surrounded with a well using a PAP pen and 20 uL of water was carefully pipetted onto the section. The section was held at 20°C, 100% humidity for 16 hours. As the section dried out after 16 hours, the experiment was repeated by surrounding the fixed section with a plastic well 1cm deep. After 16 hours in the humidity chamber at 20°C the section was still covered in solution. The section was then analysed using a SACPIC stain (section 8.3.3). It was found that the sections stained very dark green. They were stained so well, that the dark green colour obliterated all detail. The indigo carmine solution was thus diluted 1:5 in water.

The experiment was repeated using 100 uL of an enzyme solution (0.5 M carbonate buffer at pH 10.5, 0.5%v/v Teric BL8, 0.2%v/v Busan 85 and 4% v/v enzyme (Purafect)) instead of water. After 16 hours the single section was examined using the diluted SACPIC stain. It was found, however, the enzyme treatment caused changes to the skin section that resulted in it being destroyed after only a small amount of handling.

Since the sections were not fixed to a slide as in previous trials it was decided that allowing the section to fully dry (24 hours at 20°, 65% r.h.) before applying the enzyme would allow the section to become firmly attached to the glass slide, which would hopefully make it more amenable to further manipulation during staining. The experiment was repeated using a 2% v/v enzyme solution and air dried sections. It was found that while the sections were stronger, some sections dried out while others were still too fragile to stain without disintegration.

Because some of the sections dried out even using high walled wells inside the humidity chamber, 500 uL of enzyme solution was trialled using single sections. Enzyme solutions of a range of different enzyme concentrations (3, 6, 12 uL of enzyme solution in 500 uL) were therefore prepared. Inspection of the SACPIC stained sections indicated that application of 3 uL of enzyme solution in 500 uL (0.6% v/v) in buffer (0.5 M carbonate buffer at pH 10.5, 0.5%v/v Teric BL8, 0.2%v/v Busan 85) was sufficient to simulate the results observed during the application of enzyme depilatory to whole skins. However the section treated with 6 uL enzyme in 500 uL buffer was destroyed during staining. Since the sections were still particularly fragile after enzyme treatment further trials involved the use of Hamilton syringes to remove and apply different staining or washing solutions.

6.2.2.1 Chemical fixation

In an attempt to enhance the stability of the section after incubation with the enzyme solution a trial was carried out to determine if the sections could be toughened by exposing them to either alcohol or formaldehyde without affecting the SACPIC results. A section that was exposed to 70% ethanol for 10 min prior to application of the SACPIC stain was much tougher and easier to handle than a section with no treatment. Treating sections with buffered formalin solution (see section 7.2.1.2) for 180 min made no improvement to their fragility. It was therefore determined that treatment of sections with 70% ethanol after exposure to the enzyme was the best method for preparing them for staining.

6.2.3 Immunohistology of enzyme treated single sections

Earlier work showed that it was advantageous to keep collagen VI intact during enzyme depilation. The protocol developed for immunohistology in this section was based on the protocol that had been used to visualise staining due to anti-collagen VI antibodies. A problem with immunohistology, however, is that the fixation process can have implications on the ability of the primary antibody to recognise and bind to the antigen. The protocol developed for collagen VI required that the sections were cut from formalin fixed, paraffin embedded blocks. It was not possible to cut further sections from a section of skin that was already only 20 um thick. It was therefore decided that the 20 um sections would be fixed in buffered formalin solution (see section 7.2.1.2) whilst on the slide and then processed into paraffin wax by hand in order to present the

collagen immunohistological protocol with the same starting point. i.e. a formalin fixed and wax embedded section.

6.2.3.1 Single section depilation immunohistology method development

In order to be able to test the depilation activity of extremely small concentrations of specific enzymes, each section was encircled with a PAP pen to form a deep well which was then surrounded by softened paraffin wax. To this well containing the section, 100 uL of enzyme solution was added, and the section incubated at 20°C and high humidity for 16 hours without the section drying out.

The results shown are for sections exposed to buffer either with or without enzyme at a concentration equivalent to 5 ug per 100 uL. Again, due to the fact that sections are never physically fixed to the slides extreme care was required when handling the liquid changes. After 16 hours of immersion in the enzyme buffer solutions, the sections were carefully drained and treated with buffered formalin solution (see section 7.2.1.2) for 3 hours. After formalin fixation the solution was again carefully drained and the sections taken through ethanol into xylene following the protocol described in section 8.3. Once in xylene the wax wells were removed from each slide. A small piece of wax was then placed next to the section and the slide carefully heated on a warming plate until the wax was just fluid enough to flow. The wax was then drained off the slide in a direction over the section. This was repeated twice to give two changes of paraffin wax and preserve the section for later immunohistology.

6.2.3.2 Single section enzyme application method

The method development described above led to the following method which was used to examine micro-quantities of enzymes. Sections were cut from shaved raw skin at 20 um and air fixed overnight on glass slides at 20°C 65% rh. The sections were the surrounded by a plastic well 10mm high 10mm diameter. The section was then incubated at 20°C 100% rh for 16 hours with 5 uL enzyme dissolved in 500 uL of enzyme buffer; (0.5 M carbonate buffer at pH 10.5, 0.5%v/v Teric BL8, 0.2%v/v Busan 85). After exposure to the enzyme the sections were carefully drained with a Hamilton syringe and stained either with the SACPIC stain or by Immunohistology

6.2.3.3 Single section SACPIC method

After exposure to the enzyme and draining of the enzyme solution the section was exposed to 70% ethanol for 10 minutes in order to toughen it for the subsequent SACPIC staining. The SACPIC staining procedure was then carried out as described in section 8.3.3 with the exception that solutions were added and removed from the well using a Hamilton syringe rather than the section added and removed from the solutions and the Picro-indigo-carmine solution was diluted 1:5 before use. Sections were then mounted in DPX (section 8.3.5)

6.2.3.4 Single section immunohistology method

After exposure to the enzyme and draining of the enzyme solution the section was dehydrated through ethanol and xylene following the protocol described in section 8.3.1.4 with the exception that the solution was added to the section rather than the section passed through the solutions and that the paraffin was added as described in section 6.2.3.1 by melting paraffin wax on the slide and draining melted wax across the paraffin across the section. This was carried out because the section was not physically fixed to the slide and so the conventional technique of passing the slide through the appropriate solutions was not possible. The sections prepared in this way were then treated using the immunohistological method described in section 8.3.6 except that solutions were changed using a Hamilton syringe to ensure that the "free floating" sections were not lost.

6.2.4 Single section depilation

The enzymes chosen for examination using the single section depilation investigation were as follows:

Purafect - chosen because analysis of the exposure of lambskins had already been analysed using SACPIC staining, proteomic techniques, and immunohistochemistry. The results with different enzymes will therefore be able to be compared with those using Purafect.

Dispase (Sigma, D4818 -2MG) - chosen because claims had been made that this enzyme could unhair without damage to the enamel layer of the skin (Paul *et al*, 2001)

MMP-11 (Sigma, M7692-10 uG)– chosen because it is described as having specific proteolytic activities against extracellular proteins including laminin, and fibronectin, but not against collagens (Sternlicht and Werb, 1999) (see sections 2.8.2.1 and 5.6). This is an important distinction because if a protease was found with no activity against any collagen but was found to be highly active against the remainder of the extracellular proteins there would be a real possibility to release the wool fibre from the extracellular matrix without the concomitant damage of any of the collagens that go into the final product

Each enzyme was applied to the section at a concentration of 5 ug/100 uL buffer. Purafect was made up in enzyme buffer as previously described. Dispase was applied in a neutral buffer containing 50mM calcium chloride set to pH 7.4 using NaOH. MMP-11 was applied in the solution as received in its active form at 5 ug/100 uL (pH 7.0) diluted 1:1 with a solution containing 100mM calcium chloride, 100mM zinc chloride adjusted to (pH 7.0). NB: No enzymes were received as zymogens and so were applied directly in there active form including cofactors as required.

6.2.5 Results

Using the single section depilation method developed in the above sections, three different proteolytic enzymes were examined for their ability to remove the structures associated with depilation and to leave intact collagen VI behind. The results for each in comparison to control sections treated with buffer only are illustrated in Figure 105.

The untreated control section which was exposed to a buffer solution that contained no proteolytic enzyme shows fully intact structures around the wool root and at the skin surface as expected. (Controls using only water were the same – data not shown).

Carrying out SACPIC stains on the single section of skin that had been treated with Purafect showed the same form of destruction of the wool root sheath that was observed on sections cut from whole skins that had been similarly treated. Dispase also caused some destruction of the wool root confirming previous reports of its activity in skins. The Dispase treated section, however, had significantly more intact wool bulb in comparison to skins treated with Purafect indicating that it was not attacking the

proteins in those structures as required. Although Dispase may be useful in removing cells of the epidermis as previously described it would appear that the removal of wool by this enzyme may be a more difficult task. These results, therefore, may explain the findings of (Paul *et al.*, 2001) who showed that while Dispase acted as a depilatory agent it was not a complete depilatory in that some fine hairs were left behind.

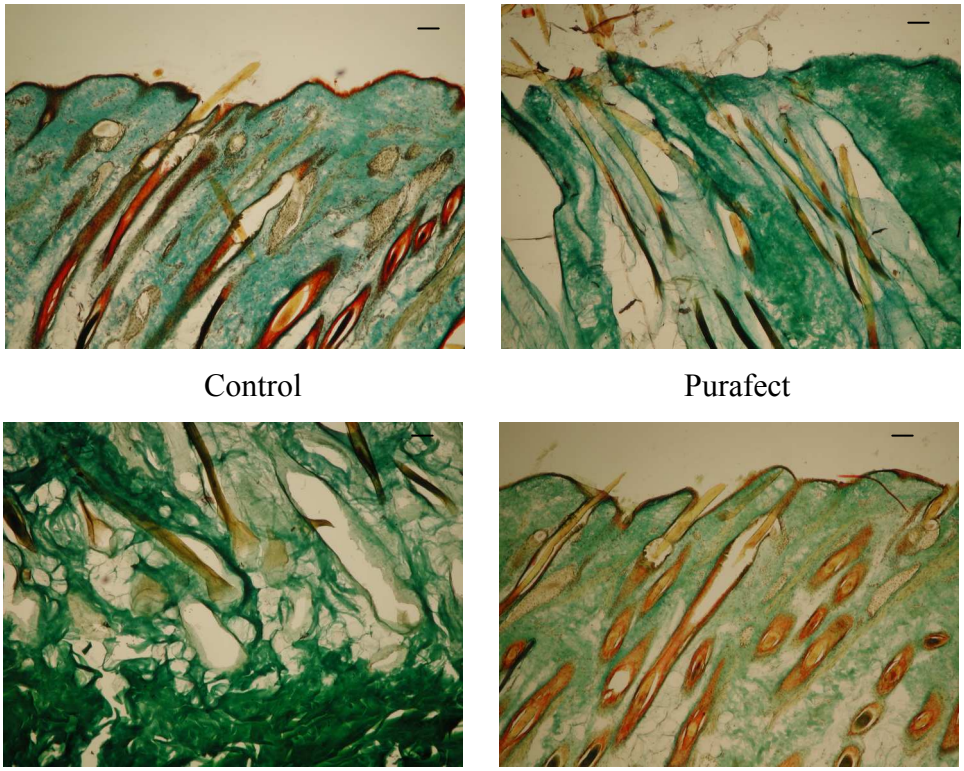


Figure 105: SACPIC stains of single section depilation micro assay: Note evidence of the processes of depilation in sections treated with Purafect or Dispase. All bars are 50 um

Sections treated with MMP-11 were not significantly different from the controls which were treated with buffer only. Specifically the structures of the wool root sheath remained predominantly intact indicating that the use of MMP-11 under these conditions would not result in successful depilation.

Additional sections that had been exposed to prospective enzyme depilatory reagents were assessed for persistence of collagen VI. The results are illustrated in Figure 106 below:

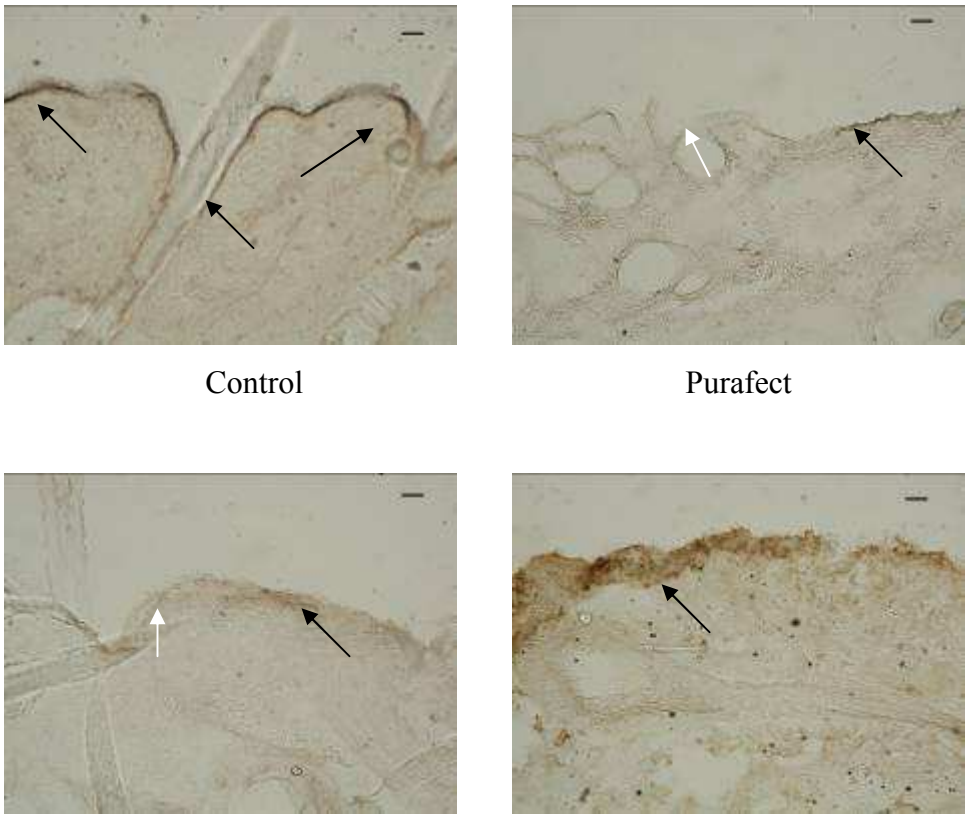


Figure 106: Collagen VI immunohistology of single section depilation micro assay: Note some collagen VI in each section (black arrows) with evidence of the removal of stained material within the grain enamel of sections treated with either Dispase or Purafect (white arrows). All bars are 25 um

The collagen VI immunohistological stain again shows intact collagen VI at the surface of the control section showing that as expected collagen VI remained in the grain enamel layer in the presence of buffer (Controls using only water were the same – results not shown).

When whole skins were treated with Purafect there was reduced staining in the grain enamel consistent with the loss of collagen VI from this area of the skin. In particular,

the regions in which the staining was absent corresponded to those regions of the skin surface that were most damaged. Specifically the surface fibres appeared frayed and did not present a smooth surface.

The skin treated with Dispase showed some damage to the collagen VI and some disruption of the grain surface in those regions. This indicates that it may have some damaging activity to collagen VI which could potentially result in a reduction in the quality of the grain surface in the finished product.

It was hoped that the MMP-11 would result in a significant degree of destruction to the structures of the extracellular matrix but without damage to collagen VI. The results show that while there was still significant immunohistological staining at the skin surface indicating that a significant amount of collagen VI remained intact, the SACPIC staining indicated that there was hardly any change to the proteins in the wool root shaft. This indicated that MMP-11 would not enhance depilation. It is possible that the use of a human (MMP-11), which is supposedly specific to human extracellular proteins, meant that it was not active against the ovine proteins. Alternatively because MMP-11 is characterised as being relatively less active in comparison to other endogenous proteases, it failed to degrade enough of the proteins under the conditions used to remove the proteins around the wool root. It is still possible that ovine MMP-11 might achieve effective depilation without affecting collagen VI. This would require the isolation of ovine MMP-11 which is outside the scope of this work. The most likely explanation for the inability of MMP-11 to degrade the proteins around the wool root is that it simply does not have a broad enough spectrum of activity.

It may be worthwhile in the future to use the techniques developed here to examine other pure, and highly specific proteases to check their activity against collagen VI and the different proteins around the wool shaft.

6.3 Controlling undesirable activity - Conclusions

The use of different salts to modify the activities of depilatory enzymes was found to result in improved skin quality depending on the enzyme or salt used. The use of

potassium sulfate in combination with Purafect produced depilated pelts with no visible damage to the grain surface. Complete depilation took three days to complete however, and it was beyond the scope of this work to determine why potassium sulfate had this effect. Future work on this process may lead to the development of an acceptable enzyme based depilation process.

A “micro” method for the examination of prospective depilatory reagents was successfully developed and used to investigate the activity profiles of different enzymes. This method may be an option for the further investigation of a range of prospective depilatory enzymes that are not available in quantities suitable for pilot scale assessment.

Application of a purified protease that ostensibly had an activity against non-collagen components of the basal layer but not collagen VI was shown not to be a suitable enzyme for depilation. Application of a selection of broader spectrum proteases with the same overall requirements would therefore be useful in the future.

Conclusions: Proteolytic depilation of lambskins

7.1 Research overview (The big picture)

The direction of the research undertaken in this work was to move towards an enzyme based system for the depilation of ovine skins. Discovering the reasons for the shortcomings in enzymatic depilation was the driving force to discover the molecular bases of the processes involved in dewooling. By seeking to understand the enzyme depilation process it was hoped that a pathway towards a successful enzyme depilatory system could be found.

Damage to the skin did not occur at the same time as the depilation process as a small window of time occurs between the depilation and grain enamel layer damage when using a non specific broad spectrum protease. Variation found naturally across the skin results in regions that become depilated and then damaged before other regions can become depilated. The time difference between the depilation and damage at a local region in the skin is orders of magnitude shorter than the time differences for depilation across the skin. Unfortunately levelling out the skin properties by removing barriers to enzyme penetration results in an acceleration of the depilation process but not an expansion of the window of opportunity for depilation without associated damage. Furthermore increasing the time taken to depilate is likely to result in an enlarged window of opportunity for depilation without associated damage but may result in industrially unacceptable processing times.

By taking a mechanistic approach to the problems with enzymatic depilation it was determined that successful enzymatic depilation is likely to be achieved only through the use of a broad spectrum protease which has no activity against any of the collagens. Whilst collagen type I is the main collagen in skin and has a structure which is quite resistant to non-specific broad spectrum proteases, it is held in place through interactions with minor collagens. Collagen VI in particular located around bundles of collagen in the surface of skin plays a role in maintaining the integrity of the collagen structure of the surface and hence the integrity of the surface of the final product. Collagen VI does not have the resistant structure that collagen I has and can be degraded by the broad spectrum depilatory enzymes used to date. Its destruction and

removal from the fine fibrils of the pelt surface result in the release and subsequent damage of the structural fibrils of the grain surface.

The key to a successful enzyme depilation system would be to use the techniques developed in this work such as the single section depilation method to assess enzymes that have a broad specificity to non-collagen proteins. Some efforts were made towards this aim but further enzyme screening work is required to assess ovine endogenous enzymes or other alternative proteolytic enzymes that may be less well characterised than those tested in this work.

7.2 Future direction of further research

While advances have been made in the understanding of the enzymatic depilatory process in terms of its deficiencies, mechanism, and requirements for success, a clear industrial process that can achieve successful enzymatic depilation, is still lacking. Due to the broad based approach required for this work it is likely that a more detailed approach focussing on detailed investigations outlined below might lead to further insights into the enzyme depilation mechanism. In order to achieve this goal further research could be carried out along a number of lines as follows:

- A plausible choice (MMP-11) for an enzyme that depilates but has no activity against collagen VI was investigated in this work. While the available MMP-11 was not sufficiently active to achieve depilation, it is likely that the techniques developed and used in this thesis could be applied to other enzymes. i.e. a selection of enzymes that are known to be active against extracellular proteins including laminin but also active against some collagens could be examined to see if their collagenase activity can be controlled enough to allow depilation without damage to the collagen structure.
- While evidence for the importance of collagen VI as a protein of interest during enzymatic depilation has been demonstrated in this work, further work could be carried out to investigate specific changes in the glycosaminoglycans or lipid complement as a result of enzymatic depilation.

- Diffusion barriers were found to be important in the depilation process. Investigation of the removal of water soluble materials showed that overall depilation efficiency could be improved. Further investigation of the non-protein barriers to enzyme penetration, particularly fats, may also further improve depilation efficiency and evenness and are more than likely to reduce processing times.
- Work carried out here showed that visibly undamaged leather was generated after three days exposure to Purafect and potassium sulfate. An investigation into how this salt protects the skin from damage during this extended depilation time may lead to the discovery of a non-damaging mechanism for the depilation of lambskins
- This research involved an investigation into proteolytic activities of specific enzymes and proteins. While this avenue of investigation is by no means exhausted it is possible that a search for an enzyme targeted cleavage recognition sites explicitly not present in collagens may yield an enzyme or suite of enzymes capable of achieving or assisting enzyme depilation without damaging the important collagens.

Appendices

8.1 Published works

Portions of the work enclosed in this thesis have been published. Details of the assistance of contributing authors are as follows:

Edmonds, R. L., Deb Choudhury, S., Haverkamp, R. G., Birtles, M., Allsop, T. F., & Norris. G. E. (2008). Using proteomics, immunohistology, and atomic force microscopy to characterize surface damage to lambskins observed after enzymatic dewooling. Journal of Agriculture and Food Chemistry, vol. 56 no. 17.:

- Deb Choudhury, S. Developed the 2D electrophoresis and MALDI TOF techniques for use on ovine skin and assisted in manuscript preparation.
- Haverkamp, R.G. Assisted in the operation of the atomic force microscope
- Birtles, M. Developed the general immunohistological techniques used and advised on technique modifications for ovine skin.
- Allsop, T.F. Assisted in a supervisory role and manuscript proofing.
- Norris, G.E. Assisted in a supervisory role and in manuscript preparation.

Edmonds, R. L. (2008). Enzyme depilation. Report of the fifty ninth annual LASRA conference Palmerston North: New Zealand Leather and Shoe Research Association.

Allsop, T., Edmonds, R., Passman, A., Deb Choudhury, S., & Norris, G. (2007). Processing changes in the structure of lambskin in relation to the quality of the leather. XXIX IULTCS Congress (p. #1). :

- Allsop, T. Assisted in a supervisory role and manuscript proofing and presented the work at the congress.
- Passman, A. Assisted in a supervisory role
- Deb Choudhury, S. Developed the 2D electrophoresis and MALDI TOF techniques for use on ovine skin and assisted in manuscript preparation.
- Norris, G.E. Assisted in a supervisory role and in manuscript preparation.

Edmonds, R., Das Gupta, S., Allsop, T., Cooper, S., Passman, A., Deb Choudhury, S., & Norris, G. (2005). Elastin in lamb pelts - its role in leather quality. XXVIII IULTCS CONGRESS Associazione Italiana Dei Chemici Del Cuoio.

- Das Gupta, S. Supervised and assisted with tanning skins.
- Allsop, T., Assisted in a supervisory role

- Cooper, S., Prepared work on location and distribution of elastin in various species and assisted with the method of estimation of elastin levels using histology.
- Passman, A. Assisted in a supervisory role
- Deb Choudhury, S., Assisted in the development of the quantitative elastin technique
- Norris, G.E. Assisted in a supervisory role

Edmonds, R. L. (2002). The effect of elastin on sheep pelt area yields. Report of the fifty third annual LASRA conference Palmerston North: New Zealand Leather and Shoe Research Association.

8.2 Standard conventional processes

8.2.1 Standard conventional painting and liming process

- Skins are painted with 300g/m^2 of a solution comprising

  140 g/l Sodium sulfide flake (~60% H_2O)

  50 g/l hydrated lime (Websters Hydrated Lime Co. Ltd, New Zealand

  5 g/l pregelled starch (Solvitose, Avebe, Holland).

  Then held over night
- Pull wool from skins, weigh the dewooled slats and place them in a processing drum
- Add 80% water (All percentages based on the weight of the dewooled skins)
- run 30 min

  adjust sodium sulfide concentration to 2% w/v
- Run overnight
- drain
- Wash 5 times with 200% water at 25°C for 20 minutes each
- drain

8.2.2 Standard delime and bate process

- 100% water at 35°C

  2% ammonium chloride (Clark products Ltd, New Zealand)

  0.05% pancreatic bate (Tanzyme, Tryptec Biochemicals, New Zealand)
- Run 75 min

- Drain
- Wash 3 times with 100% water at 25°C for 20 minutes each

8.2.3 Standard pickle

- Add 90% water
  Add 20% common salt (Grade 23 medium solar salt, Pacific salt, New Zealand)
- run 10 min
- Add 10% water
  Add 2% sulfuric acid (Clark products Ltd, New Zealand)
  adjust to pH <3.0 using formic acid (Jasol, New Zealand) diluted 1:10 if necessary
- run 3 hours.

8.2.4 Aqueous degrease

- 100% water at 35°C
  4% non-ionic detergent (Tetrapol LTN, Shamrock, New Zealand)
- run 90 minutes
- Drain
- 5x 100% washes at 25°C for 15 minutes each

8.2.5 Chrome tannage

- 100% water at 35°C Percentages based on weight of pickled pelts
  8% common salt
  1% di-sodium phthalate (Feliderm DP, Clariant, United Kingdom)
  1% formic acid
- run 10 minutes
- Add 5% Chrome sulfate powder (33% basic) (chromosal B, Lanxess, Germany)
- run 30 minutes
- Add 0.6% Magnesium oxide (Acros, USA)
  Heat to 40°C
  Run overnight
- Drain
- 300% water at 25°C

- run 10 minutes

8.2.6 Retan to crust leather

- 150% water at 25°C
  1% sodium formate (Celanese Ltd, USA)
  0.4% sodium bicarbonate (Qingdao soda ash industry Co. Ltd, China)
  run 45 minutes
- 6% Syntan (Tanicor PWp, Clariant, United Kingdom)
- run 30 minutes
- Drain
- 300% water at 50°C
  run 10 minutes
- Drain
- Add 100% water at 60°C
  0.2% Ammonia solution (30% w/w) (Scharlau chemie, Spain)
- Add 20% water at 25°C
  5% fat liquor (3 parts chromopol UFB/W, 7 parts Coripol ZCK, Shamrock, New Zealand)
  0.5% Formic acid (diluted 1:10 in water)
- run for 90 minutes
- Drain
- 1x 300% washes at 25°C for 30 minutes

8.3 Stain solutions

8.3.1 Section preparation

Section were cut from either cryostat frozen material, formalin fixed, Bouin's fluid fixed paraffin embedded, or formalin fixed paraffin embedded samples. The sample preparation for each is given below

8.3.1.1 Cryostat frozen samples

Samples were obtained as fresh as possible for the process and snap frozen in isopentane that had been pre-cooled with liquid nitrogen. Samples were kept in a frozen state until sections were cut.

8.3.1.2 Formalin fixation

Samples that did not contain acid (all samples except those of pickled pelt) were placed in a solution of buffered formalin for a minimum of 24 hours. Pickled pelt samples were fixed in formalin in brine

8.3.1.2.1 Standard buffered formalin solution

0.0375 M Phosphate buffer

1.3% w/v di-sodium hydrogen phosphate

(#100-5035.500, Pure science, New Zealand)

0.9% w/v sodium di-hydrogen phosphate di-hydrate (Sharlau chemie, Spain)

Adjusted to pH 6.65

0.8%w/w formaldehyde (37% formaldehyde solution, Jasol, New Zealand)

8.3.1.2.2 Buffered formalin in brine solution

0.0375 M Phosphate buffer

1.3% w/v di-sodium hydrogen phosphate

0.9% w/v sodium di-hydrogen phosphate di-hydrate

1% w/v calcium chloride (LOBA chemie, India)

5% w/v sodium chloride (#100-1013.1000, Pure science, New Zealand)

Adjusted to pH 6.65

0.8%w/w formaldehyde

8.3.1.3 Bouin's fluid fixation

75% Saturated picric acid (1.2% solution, Applichem, Germany)

25% Formalin (37% solution)

5% Acetic acid (Lab-scan Asia co. Ltd, Thailand)

8.3.1.4 Paraffin embedding protocol

Samples were passed through alcohol into paraffin by exposing the samples to the following protocol:

70% Ethanol	30 min (Absolute ethanol, Ajax fine chemicals, Australia)
95% Ethanol	30 min
100% Ethanol	30 min x 3
Xylene	30 min x 2 (Lab-scan Asia co. Ltd, Thailand)
Paraffin wax (60°C)	30 min x 3

8.3.1.5 De wax protocol

Section to be de waxed were treated to the following protocol

Xylene	5 min
Xylene	5 min
100% ethanol	3 minutes
100% ethanol	3 minutes
95% ethanol	1 minute
70% ethanol	1 minute
Distilled water	

8.3.2 Phosphate buffered saline 0.01M (PBS)

4.54 g anhydrous di-sodium hydrogen phosphate

1.09 g anhydrous sodium di-hydrogen phosphate

32 g sodium chloride

4 L water

Check pH 7.2

8.3.3 SACPIC staining method

8.3.3.1 Celestin blue

10g Iron alum (Ammonium ferric sulfate dodecahydrate, $NH_4Fe(SO_4)_2 \cdot 12H_2O$) (BDH chemicals Ltd, England)

Dissolved in 200mL RO water

Mix overnight

Add 1.0g Celestin blue powder (Fluka, Switzerland)

Heat slowly in flask (watching for froth)

Boil for 3 min

Cool

Filter into 250mL container

Add 6.8mL Glycerol (#100-GLEP.2500, Pure science, New Zealand)

Shake well

8.3.3.2 Scott's tap water

2 g/L Sodium bicarbonate

20 g/L Magnesium Sulfate

8.3.3.3 Safranine solution

Add the following ingredients in order and mix for one hour

2g Safranine (BDH Chemical Ltd, England)

49mL Absolute ethanol

49mL RO water

8.3.3.4 Picric acid in ethanol

0.5 ML Picric acid (Saturated aqueous solution)

50mL Absolute alcohol

mix

8.3.3.5 Picro-indigo-carmine

0.125g Indigo Carmine powder (Sigma, USA)

50mL Picric acid (Saturated aqueous solution)

mix

8.3.3.6 SACPIC staining protocol

Water rinse	
Celestin blue solution	5 min
Water rinse	
Haematoxylin	5 min
(Mayers Hamalaun solution, Applichem, Germany)	
Water rinse	
Scott's "tap water"	2 min
Water rinse	
Safranine solution	5 min
70% Ethanol	
Absolute ethanol	
Picric acid in ethanol	2 min
95% Ethanol	

70% Ethanol

Water rinse

Picro-Indigo-Carmine 1 min

Water rinse

8.3.4 Alcian blue periodic acid Schiff stain protocol

Water rinse	6 × 2	min
1mL 3% acetic acid + 1 drop Alcian blue	30	min
Water rinse	6 × 2	min
1% periodic acid (BDH chemicals Ltd, England)	20	min
Water rinse	6 × 2	min
Schiff's reagent (Sigma, USA)	15	min
Water rinse	6 × 2	min
Mayers haematoxylin	5	min
Water rinse	10 × 1	min

Mount using DPX (section 8.3.5)

8.3.5 Mounting protocol

70% alcohol	4 × 2	min
Absolute alcohol	4 × 2	min

Xylene

Mount in DPX (Ajax fine chemicals, Australia)

8.3.6 Immuno-histological stain protocol

De-wax sections (section 8.3.1.5)

Epitope retrieval (if any - section 8.3.7)

Equilibrate in phosphate buffered saline (PBS 8.3.2) 1 min

Endogenous peroxidase wash (3% peroxide in PBS 8.3.2) 30 min

Equilibrate in phosphate buffered saline (PBS 8.3.2) 3x1 min

Encircle the section with a PAP pen to create a fluid barrier to hold the reagents

Block non-specific binding sites:

1% bovine serum albumin (BSA) 5 min

Drain test slides

Apply antiserum diluted in 1% BSA in (PBS 8.3.2) 1 hr

Equilibrate in phosphate buffered saline (PBS 8.3.2) 3x1 min

Biotinylated secondary antiserum:

Diluted 1:200 in 1% BSA in (PBS 8.3.2) 30 min

(either Amersham anti-mouse from sheep RPN1001, or Amersham anti-rabbit from sheep RPN1004 depending on which primary antibody was used as specified by the manufacturer)

Equilibrate in PBS(8.3.2) 3x1 min

biotin-streptavidin-peroxidase preformed complex (Amersham biotin-streptavidin-peroxidase RPN1.034 diluted 1:200 in BSA). 15 min

Equilibrate in PBS (8.3.2) 3x1 min

Peroxidase histochemistry:

0.4 mg/mL 3,3 diaminobenzidine (DAB) made fresh with the addition of 2 uL/mL 30% peroxide

Sections were then monitored for 3 minutes until colour developed at which point the reaction was quenched by immersing the slide in PBS

Slides were then counter stained in Meyer's haemalum

Rinse in tap water

Mount in DPX

8.3.7 Epitope/Antigen retrieval methods

Three epitope or antigen retrieval methods were investigated in an attempt to visualise a selection of proteins to which primary antibodies were available within this project. Those were – no epitope retrieval, trypsin epitope retrieval, and citrate buffer epitope retrieval.

8.3.7.1 Citrate buffer epitope retrieval

8.3.7.1.1 Citrate buffer solution

Tri-sodium citrate di-hydrate 2.94 g (Pancreac, Spain)

Distilled water 1000 mL

Adjust to pH 6.0 using HCl

Stored at room temperature for no more than 1 month

8.3.7.1.2 Citrate buffer epitope retrieval method

Sections were covered with citrate buffer solution (8.3.7.1.1) at 95°C for 30 minutes (10 minutes and 30 minutes were also trialled but were not successful for any antibody tested)

Sections were removed from the citrate buffer solution and allowed to cool to room temperature for 20 minutes.

Sections were then equilibrated in PBS

8.3.7.2 Trypsin epitope retrieval solutions

The trypsin epitope retrieval method used in this work is based on that described on the Immunohistochemistry world web site (IHCworld, 2007).

8.3.7.2.1 Trypsin stock solution (0.5% in distilled water)

Trypsin	50 mg (Trypsin 3703 TRC, Worthington biochemical Corp, USA)
Distilled water	10 mL

Stored at -20°C for no more than 1 month

8.3.7.2.2 Calcium chloride stock solution (1% in distilled water)

Calcium chloride (di-hydrate)	100 mg
Distilled water	10 mL

Stored at 4°C for no more than 1 month

8.3.7.2.3 Trypsin working solution (0.05% in distilled water)

Trypsin stock solution (8.3.7.2.1)	1 mL
Calcium chloride stock solution (8.3.7.2.2)	1 mL
Distilled water	8 mL

Adjusted to pH 7.8 using 1M NaOH and 1M HCl

Stored at 4°C for no more than 1 month

8.3.7.2.4 Trypsin epitope retrieval method

Sections were covered with trypsin working solution (8.3.7.2.3) at 37°C for 15 minutes (10 minutes and 20 minutes were also trialled but were not as successful for any antibody tested)

Sections were removed from the trypsin solution and allowed to cool to room temperature for 10 minutes.

Sections were then equilibrated in PBS

8.4 Polyacrylamide gel methods

A range of gels for electrophoresis with both isocratic and gradient concentrations were prepared.

The formulations for typical isocratic and typical gradient gels are given below with different polyacrylamide concentrations achieved by adjusting the relative amounts of 40% acrylamide stock and water

All gels prepared were either small format (8cm x 10cm x 0.75 mm) or large format (18 cm x 21 cm x 0.75 mm).

8.4.1 Isocratic gel (7.5% Polyacrylamide, 2 small format gels, example)

Milli Q water 5.425 ML

Acrylamide-Bis Acylamide (40% solution) 1.875 mL
(BioRad, 161-0148, USA)

Tris buffer (1.5 M, pH 8.8) 2.5 mL
(Tris X, GERBU biotechnik GmbH, Germany)

Ammonium persulfate (10% solution) 100 uL
(BioRad, 161-0700, USA, Prepared fresh, from dry stock stored under vacuum)

Sodium dodecyl sulfate (10% solution) 100 uL
(BioRad, 161-0301, USA)

TEMED 8 uL
(BioRad, 161-0800, USA)

8.4.2 Gradient gel (7 – 14 % Polyacrylamide, 2 small format gels, example)

Half the volume of gel to be poured was made from the light fraction (below) formulation and the other half was prepared from the heavy fraction (below) formulation. The two formulations were then mixed on a linear gradient mixer to

produce a linear gradient between the two. A pump was used to pump the mixture from the gradient mixer which was then carefully poured into a gel from the bottom of the gel working up. The presence of sucrose in the heavy fraction helped the layering of the polyacrylamide with the concentration going from that of the heavy fraction to that of the light fraction.

8.4.2.1 Light fraction

Milli Q water	2.840 mL
Acrylamide (40% solution)	1.093 mL
Tris buffer (1.5 M, pH 8.8)	1.333 mL
Ammonium persulfate (10% solution)	48 uL

(Prepared fresh, daily, from dry stock stored under vacuum)

Sodium dodecyl sulfate (10% solution)	53 uL
TEMED	2 uL

(Actually TEMED was 1 uL added directly to the gradient mixer column)

8.4.2.2 Heavy fraction

Milli Q water	1.661 mL
Acrylamide (40% solution)	2.582 mL
Tris buffer (1.5 M, pH 8.8)	1.666 mL
Ammonium persulfate (10% solution)	28 uL

(Prepared fresh, daily, from dry stock stored under vacuum)

Sucrose	0.96 g

(Serva Feinbiochemica GmbH Co. Ka, Germany)

Sodium dodecyl sulfate (10% solution)	64 uL
TEMED	2 uL

(Actually 1 uL TEMED was added directly to the gradient mixer column)

8.4.3 Ornstein Davis native gel

Poly acrylamide gels were prepared using the following formulation:

1.34×(X%) mL Bisacrylamide (2% solution)

(BioRad, 161-0142, USA)

2.43×(X%) mL Acrylamide (40% solution)

(sigma A-4058, USA)

3.58 mL Water

2.5 ML Resolving buffer
1.5 M Tris-HCl at pH 8.8
25 uL TEMED
200 uL Ammonium persulfate solution (10%w/v) Where X was the desired gel concentration.

8.4.4 Low pH native PAGE gel

Poly acrylamide gels were prepared using the following formulation:

1.34×(X%) mL bisacrylamide (2% solution)
2.43×(X%) mL acrylamide (40% solution)
3.58 mL water

2.5 ML resolving gel buffer
KOH/acetic acid buffer at pH 4.3
48 mL 1M KOH
72 mL glacial acetic acid
34.8 mL water

1.25 ML riboflavin 5 phosphate solution (light activated cross linking catalyst) 4mg/100mL

Where X was the desired gel concentration. The gels were then exposed to light for 1 hour to enable polymerisation.

8.5 Polyacrylamide electrophoresis

8.5.1 Sample extraction buffer

Although a range of sample extraction techniques exist. Skin contains a range of hydrophobic proteins and as such is particularly difficult to dissolve. Difficulties in dissolving such proteins can some times be overcome with the use of reducing agents and strong chaotropic agents such as Urea, Thiourea, dithiothreitol, and CHAPS (Berkelman & Stenstedt, 1998); (Mujahid *et al.*, 2007).

The following sample extraction buffer was therefore used

7 M	Urea
2 M	Thiourea
4% w/v	CHAPS
40 mM	Dithiothreitol
30 mM	Tris (base only)

(Complete mini™ was also added according to the manufacturers instructions 1 pellet per 10mL)

Approximately 20 ug of freshly cut skin sample was used to generate a sample extract.

8.5.2 Chloroform-Methanol precipitation method

When proteins are extracted using the method applied in this work, the salts that are used can interfere with the electrophoresis and therefore need to be removed (Berkelman & Stenstedt, 1998). A range precipitation methods are available to carry this out such as trichloroacetic acid, acetone, chloroform/methanol, ultra filtration (Jiang *et al.*, 2004), cold acetone precipitation and TCA precipitation. The following chloroform methanol precipitation procedure was used because of its rapidity, reproducibility, and ease of application (based on (Wessel & Flügge, 1984)).

	200 uL	Extracted sample
Add	400 uL	Methanol (Pancreac, Spain)
	Mix by vortexing for 10 seconds	
	Centrifuge at >10,000 *g* for 10seconds	
Add	100 uL	Chloroform
	Mix by vortexing for 10 seconds	
	Centrifuge at >10,000 *g* for 10 seconds	
Add	300 uL	distilled water
	Mix by vortexing for 60 seconds	
	Centrifuge at >10,000 *g* for 5 minutes	
	Carefully remove upper phase and discard (~800 uL)	
Add	300 uL	methanol
	Mix by vortexing for 10 seconds	
	Carefully drain the supernatant and dry the pellet under vacuum.	

8.5.3 1D Polyacrylamide sample buffer

Protein pellets prepared using techniques described in section 8.5.1, and 8.5.2 were rehydrated in the following sample buffer before being diluted 1:1 in distilled water boiled for 4 minutes and run one 1D SDSPAGE gels

15% w/v	Glycerol
2% w/v	Dithiothreitol
0.005% w/v	Bromophenol blue
6% w/v	Sodium dodecyl sulfate
0.125 M	Tris/HCl at pH 6.7

8.5.4 10x SDS tank buffer

When carrying out SDS PAGE electrophoresis the following buffer was prepared in advance and diluted 10x before use in the buffer tank of the electrophoresis equipment.

250 mM	Tris base
1.92 M	Glycine
SDS	1% w/v
Water to volume	

8.6 2D Polyacrylamide electrophoresis

8.6.1 Sample preparation

Protein pellets were prepared from samples using the techniques described in section 8.5.1, and 8.5.2.

The pellets were then solubilised in rehydration solution

2 M Thiourea,

8 M Urea

20mM DTT

0.5% v/v IPG buffer, pH 4-7

a trace of Bromophenol blue

2% CHAPS

Complete Mini™

Active rehydration of the strips was carried out at 30 Volts (V) for 10 h followed by isoelectric focusing using the following programs:

a) For 7 cm strips: 300 V step'n hold for 3 h, 1000 V gradient for 30 min, 5000 V gradient for 90 min and a final focusing step at 5000 V step'n hold for 3 h.

b) For 18 cm strips: 500 V step'n hold for 1 h, 1000 V gradient 1 h, 8000 V gradient 3 h, 8000 V step'n hold for 3 h.

The focused IPG strips were then equilibrated in two volumes of equilibration buffer (50mM Tris buffer, pH 8.8, containing 6 M urea, 30% w/v glycerol, 2% w/v SDS and a trace of bromophenol blue); 10 mL containing 10 mg/mL DTT, for 15 min followed by 10 mL containing 25 mg/mL iodoacetamide for a further 15 min. The second dimension electrophoresis was carried out either on a BioRad Mini-PROTEAN® 3 system for 7cm strips or BioRad PROTEAN II xi system for the 18 cm strips. Acrylamide gels (0.75 mm thick, 7.5% acrylamide) were cast without wells and the IPG strips laid gently on top of the gels then sealed in place with 1 % agarose. Electrophoresis was carried out at 100 V for 15 min, and then increased to a constant 200 V until the end of the run. Proteins were stained using silver (Berkelman & Stenstedt, 1998).

8.7 Poly acrylamide gel staining

The gels were first fixed then stained using either a silver stain or Coomassie blue. Coomassie blue was generally used as a quick method to determine if significant levels of protein were present, for Western blotting for example. Silver staining was carried out when higher levels of detection were required.

8.7.1 Fixatives

8.7.1.1 SDS PAGE fixative

50% Ethanol

10% Acetic Acid

8.7.1.2 Native PAGE fixative

30 % Methanol

10 % Trichloroacetic acid (TCA) (Park scientific Ltd, United Kingdom)

4 % Sulfosalicylic acid(Mallinckrodt Inc., USA)

8.7.2 Silver stain

8.7.2.1 Sensitising agent

0.02 % Sodium thiosulfate

8.7.2.2 Silver stain

10 mM Silver nitrate

8.7.2.3 Developer

6 % Sodium carbonate (anhydrous)

0.05% Formalin (37% solution)

2 % Sensitising agent (8.7.2.1)

8.7.2.4 Stop solution

5 % Acetic acid (Ice cold)

8.7.2.5 Silver staining protocol

50mL	Fixative	30 min
	Distilled water rinse	3 x 5 min
50 mL	Sensitising agent	2 min
	Distilled water rinse	1 x 5 min
50 mL	Silver stain	20 min
	Distilled water rinse	2 x 20 seconds
50 mL	Developer	until bands appear (10 minutes)
50 mL	Stop solution	10 minutes
	Distilled water rinse	2 x 20 seconds

8.7.3 Coomassie blue stain

Fixative

10 % Trichloroacetic acid (TCA)

Stain

0.05% Coomassie Blue R-250 (Bio Rad 161-0400)

50 % Methanol

10 % Acetic acid

Destain

10 % Methanol

10 % Acetic acid

The following protocol was carried out for coomassie blue staining

50 mL	10 % TCA solution	10 minutes	(Native PAGE gels only)
100 mL	1 % TCA solution	2 hours	(Native PAGE gels only)
100 mL	Coomassie blue stain	1 hour.	
100 mL	Destain	1 hour	
100 mL	Destain	Overnight	

8.7.4 Western Blotting

Western Blotting techniques were based on those described in Amersham ECL Western Blotting detection reagents and analysis system ((GE Healthcare UK Limited, 2006))

8.7.4.1 10x Transfer Buffer

Tris Base 58.1g

Glycine 29.3g

Milli Q water to make up to 1L

8.7.4.2 1x Transfer Buffer

10x Transfer Buffer 75 mL

Isopropanol 150 mL (Propan-2-ol, Lab-Scan Asia Co. Ltd., Thailand)

Milli Q water to make up to 750 mL

8.7.4.3 10x Phosphate Buffered saline for Western (10x PBS_W)

NaCl 80 g

KCl 2 g (#100-1035.500, Pure science, New Zealand)

Na2HPO4.7H20 26.8 g

KH2PO4 2.4 g (Ajax fine chemicals, Australia)

Make up to 800 mL

pH adjusted to 7.4 using HCl

Make up to 1000 mL

To this Tween 20 was added at a rate of 0.5% v/v

8.7.4.4 1x Phosphate Buffered saline for Western (1x PBS_W)

1 mL 10x PBSW (8.7.4.3)

9 mL distilled water

Gives 0.01 M Phosphate buffer at pH 7.4

8.7.4.5 BSA diluent for Western (BSAW)

1% BSA (Sigma) in 1x PBSW (8.7.4.4)

8.7.4.6 Western transfer protocol

SDS PAGE gels containing proteins were transferred to nitrocellulose using a BioRad Mini trans-blot® cell unit with the following method:

Pieces of Whatman filter paper were cut to the shape of the gel to be transferred and piled in two stacks approximately 3mm high.

A piece of nitrocellulose membrane (BioRad cat# 162-0116 0.45 um) was cut to the size of the gel.

One set of filter papers were soaked well in 1x transfer buffer (8.7.4.2) and placed on a fibre pad. The nitrocellulose piece was then soaked in 1x transfer buffer (8.7.4.2) and placed on the soaked filter papers. The gel to be transferred was then soaked in 1x transfer buffer (8.7.4.2) and placed on the nitrocellulose. One filter paper from the second stack was then soaked in 1x transfer buffer (8.7.4.2) and placed on top of the gel. A clean pipette tip was then used to carefully squeeze out any bubbles between the nitrocellulose and the gel. Next, the remainder of the second stack of filter papers was soaked in 1x transfer buffer (8.7.4.2) and placed on top. Finally another fibre pad was placed on the top to complete the transfer sandwich and the sandwich was placed in the mini transblot cell unit with the negative electrode on the gel side of the sandwich.

The transfer was run at 100V for 1.75 hours and the final transfer assessed by inspection of the nitrocellulose for pre-stained markers from the gel.

8.7.4.7 Western immuno probe protocol

The nitrocellulose membrane was treated to the following protocol

Block in BSA_W (8.7.4.5)	1 hr
Wash in PBS_W (8.7.4.4)	3 x 5 min

Incubate with primary antibody diluted 1:3333 in BSA_W (8.7.4.5)

Anti-collagen VI raised in rabbit (Chemicon AB7822)	1 hr
Wash in PBS_W (8.7.4.4)	3 x 5 min
Incubate with biotinylated secondary antibody diluted 1:3333 in BSAW (8.7.4.5)	
Amersham anti-rabbit from sheep RPN1004	1 hr
Wash in PBS_W (8.7.4.4)	3 x 5 min
Incubate with biotinylated secondary antibody diluted 1:3333 in BSA_W (8.7.4.5)	
Amersham anti-rabbit from sheep RPN1004	1 hr
Wash in PBS_W (8.7.4.4)	3 x 5 min
biotin-streptavidin-peroxidase preformed complex (Amersham biotin-streptavidin-peroxidase RPN1.034 diluted 1:3333 in BSA_W (8.7.4.5)).	1 hr
Wash in PBS_W (8.7.4.4)	3 x 5 min

8.7.4.8 Western staining protocol

The nitrocellulose membrane, which had been probed (section 8.7.4.7), was revealed using the following chemi-luminescent method (BM Chemiluminescence blotting substrate (POD) Roche cat 1500694)

10 mL Substrate solution A

100 uL Substrate solution B

are mixed and allowed to come to room temperature. The membrane is then incubated in the premixed solution for 1 minute and then pictures are then taken of the result using a Fujifilm Intelligent darkbox II

8.8 Document statistics

This document contains 91530 words in 342 pages. Thanks for reading ☺.

References

Addy, V. L. (2000). Unravelling the unhairing process. World Leather, **16**(6), 59-60.

Al-Amoudi, A., Dubochet, J., & Norlén, L. (2005). Nanostructure of the epidermal extracellular space as observed by cryo-electron microscopy of vitreous sections of human skin. Journal of Investigative Dermatology., **124**(4), 764-777.

Alexander, K. T. W. (1987). Development of a 24 hour raw to wet blue process for bovine stock. BLMRA Journal, **30**(3), 30-33.

Alexander, K. T. W. (1988). Enzymes in the tannery; catalysts for progress? - 1988 John Arthur Wilson Memorial Lecture. World Leather, **1**(6), 38.

Alexander, K. T. W., Haines, B. M., & Walker, M. P. (1986). Influence of proteoglycan removal on opening up in the beamhouse. Journal of the American Leather Chemists Association., **81**, 85-102.

Alexander, K. T. W., Walker, M. P., Webster, R. M., Stanley, A. M., Bugby, A., & Smith, Y. (1991). Selective use of enzymes during leather processing. 21st IULTCS Congress Proceedings BLC Leather Technology Centre.

Allsop, T. F. (2007). Rationalised lamb pelt processing - an update. Report of the 58th Annual LASRA conference New Zealand Leather and Shoe Research Association.

Allsop, T. F., & Passman, A. (2003). Porosity measurement as a means of determining the degree of processing of pelts. Journal of the Society of Leather Technologists and Chemists., **87**, 49-53.

Allsop, T., Cooper, S., DasGupta, S., Birtles, M., Edmonds, R., & Passman, A. (2005). Elastin in lamb pelts. Its role in leather quality. XXVIII IULTCS Congress proceedings Italy.

Allsop, T., Edmonds, R., Passman, A., Deb Choudhury, S., & Norris, G. (2007). Processing changes in the structure of lambskin in relation to the quality of the leather. XXIX IULTCS Congress (p. #1).

Allsop, T., & McClelland G. (1999). Controlling lamb pelt degreasing. Report of the fiftieth Annual Conference for fellmongers and hide processors and leather technologists (p. 71). Palmerston North: New Zealand Leather and Shoe Research Association.

Andreeva, N. S., Bochkarev, A., & Pechik, I. (1995). A new way of looking at aspartic proteinase structures: A comparison of pepsin structure to other aspartic proteinases in the near active site region. K. Takahashi (Editor), Aspartic proteinase: Structure, function, biology, and biomedical implications (pp. 19-32). New York: Plenum Press.

Aravindhan, R., Saravanabhavan, S., Rao, J. R., Nair, B. U., Thanikaivelan, P., & Chandrasekaran, B. (2004). A bio-driven lime and pickle free tanning paves way for greener garment leather production. Journal of the American Leather Chemists Association., **99**, 53-66.

Argraves, W. S. (1999). Part 3. Fibulins. T. Kreis, & R. Vale (Editors), Guidebook to the Extracellular matrix, anchor, and adhesion proteins. Oxford: Oxford University Press.

Aumailley, M., Speks, U., & Timpl, R. (1991). Cell adhesion to type VI collagen. Biochemical Society Transactions., **19**, 843-847.

Bailey, A. J. (1991). Procter memorial lecture collagen - Nature's framework in the medical, food and leather industries. Journal of the Society of Leather Technologists and Chemists., **76**, 111-127.

Bailey, A. J., & Paul, R. G. (1997). Collagen; A not so simple protein. Vol. 24. 23rd IULTCS England.

Baldwin, R. L. (1996). How Hofmeister ion interactions affect protein stability. Journal of biophysics., **71**, 2056-2063.

Bankus, J. M., & Bond, J. S. (2001). Some commercially available proteases. R. J. Beynon, & J. S. Bond (Editors), Proteolytic enzymes a practical approach. Oxford: IRL Press at Oxford Press.

Barrett, A. J., (2001). Proteolytic enzymes: nomenclature and classification. R. J. Beynon, & J. S. Bond (Editors), Proteolytic enzymes a practical approach. Oxford: IRL

Press at Oxford Press.

Bayliss, M. T. (1984). Proteoglycans: structure and molecular organisation in cartilage. D. W. L. Hukins (editor), Connective tissue matrix. London: Macmillan Press.

Bekou, V., Thoma-Uszynski, S., Wendler, O., Uter, W., Schwietzke, S., Hunziker, T., Zouboulis, C. C., Schuler, G., Sorokin, L., & Hertl, M. (2005). Detection of laminin 5-specific auto-antibodies in mucous membrane and bullous pemphigoid sera by ELISA. Journal of Investigative Dermatology., **124**(4), 732-740.

Bell, G. I. (1988). Models of cell adhesion involving specific binding. P. Bongrand (Editor), Physical basis of cell-cell adhesion (pp. 228-258). Boca Raton: CRC Press.

Berkelman, T., & Stenstedt, T. (1998). 2-D Electrophoresis, Using Immobilized pH Gradients, Principles and Methods. (Amersham Biosciences; 80-6429-60 Rev-A, 10/98).

Berla Thangam, E., & Nagarajan, T. S. R. G. (2001). Application of alkaline protease isolated from *Alcaligenes Faecalis* for enzymatic unhairing in tanneries. Journal of the American Leather Chemists Association., **96**(4).

Bettelheim, F. A. (1971). Structure and hydration of mucopolysaccharides. H. R. Elden (Editor), Biophysical properties of the skin: Volume 1 in the series, A treatise of skin. New York: Wiley-Interscience.

Bienkiewicz, K. (1983). Physical chemistry of leather making. Malabar, Florida: Robert E. Krieger Publishing Company.

Bienkiewicz, K., St Piniak, & Pawlak, J. (1983). Amine unhairing. Atti del XVIII congresso IULTCS.

Blažej, A., Galatík, A., Krul, Z., & Mládek, M. (1989). Atlas of microscopic structures of fur skins 1. Amsterdam: Elsevier.

Blundell, T. L., Guruprasad, K., Albert, A., Williams, M., Sibanda, B. L., & Dhanaraj, V. (1998). The aspartic proteinases: An historical overview. N. G. James (Editor), Aspartic proteinases: Retroviral and cellular enzymes (Vol. 436). New York: Plenum Press.

Boast, D. A., Horne, M., & Fuchsmann, R. D. (1989). Enzyme assisted unhairing: The BLMRA 24 hour soak to wet-blue process. LIRI Research Bulletin, **976**.

Böckle, B., Galunsky, B., & Müller, R. (1995). Characterisation of a keratinolytic serine protease from *Streptomyces pactum* DSM 40530. Applied Environmental Microbiology., **61**(10), 3705-3710.

Bode, W. (2002). Structural basis of matrix metalloproteinase function. Biochemical society symposium 70 London: Portland press.

Boenisch, T. E. (2001). Immunochemical staining methods 3rd edition. Carpintaria, California: DAKO Corporation.

Bongrand, P. (1988). Intermolecular forces. P. Bongrand (Editor), Physical basis of cell-cell adhesion (pp. 1-38). Boca Raton: CRC Press.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry., **72**, 248-254.

Brady, D., Duncan, J. R., & Russell, A. E. (1987). Wool fibre degradation and sheep skin depilation by extracellular enzymes produced from *Streptomyces* cultures. LIRI Research Bulletin, **931**.

Brady, D., Duncan, J. R., & Russell, A. E. (1989a). Enzyme depilatory studies part I. Review of enzyme depilation. LIRI Research Bulletin, **978**.

Brady, D., Duncan, J. R., & Russell, A. E. (1989b). Enzyme depilatory studies part III. A model for proteolytic depilation of skins. LIRI Research Bulletin, **982**.

Brady, D., Duncan, J. R., & Russell, A. E. (1990). A model for proteolytic depilation of skins. Journal of the American Leather Chemists Association., **85**(9), 334.

Briggaman, R. A., & Wheeler, C. E. (1975). The epidermal-dermal junction. Journal of Investigative Dermatology., **65**, 71-84.

Brodsky, B., & Eikenberry, E. F. (1982). Characterization of fibrous forms of collagen. Methods in Enzymology., **82**, 127-174.

Bronco, S., Castiello, D., D'Elia, G., Salvadori, M., Seggiani, M., & Vitolo, S. (2005). Oxidative unhairing with hydrogen peroxide: Development of an industrial scale process for high quality upper leather. Journal of the American Leather Chemists Association., **100**(2), 45-53.

Burgeson, R. E. (1987). Type VII Collagen. R. Mayne, & R. E. Burgeson (Editors), Biology of extracellular matrix: A series - Structure and function of collagen types. Florida: Academic Press.

Burgeson, R. E., Lunstrum, G. P., Rokosova, B., Rimberg, C. S., Rosenbaum, L. M., & Keene, D. R. (1990). The structure and function of type VII Collagen. R. Fleischmajer, B. R. Olsen, & K. Kühn (Editors), Structure, molecular biology, and pathology of collagen (Vol. 580pp. 32-43). New York: New York Academy of Sciences.

Burton., D. (1958). The methods and mechanisms of unhairing. Journal of the Society of Leather Technologists and Chemists., **42**(12), 403-414.

Cantera, C. S. (2001a). Hair-saving unhairing process Part 1. Epidermis and the characteristics of bovine hair. Journal of the Society of Leather Technologists and Chemists., **85**(1), 1-5.

Cantera, C. S. (2001b). Hair-saving unhairing process Part 2. Immunisation phenomenon. Journal of the Society of Leather Technologists and Chemists., **85**(2), 47-51.

Cantera, C. S. (2001c). Hair-saving unhairing process Part 3. "Cementing substances" and the basement membrane. Journal of the Society of Leather Technologists and Chemists., **85**(3), 93-99.

Cantera, C. S. (2001d). Hair-saving unhairing process Part 4. Remarks on the evolution of the investigations on enzyme unhairing. Journal of the Society of Leather Technologists and Chemists., **85**(4), 125-132.

Cantera, C. S., Garro, M. L., Goya, L., Barbeito, C., & Galarza, B. (2005). The diffusion barrier within the unhairing process. World Leather, **18**(6), 29-32.

Cantera, C. S., Goya, L., Galarza, B., Garro, M. L., & Lopez, L. M. I. (2003). Hair saving unhairing process. Part 5 characterisation of enzymatic preparations applied in

soaking and unhairing processes. Journal of the Society of Leather Technologists and Chemists., **87**(2), 69-77.

Cantera, C. S., Goya, L., Garcia, R., & Sofia, A. (1997). Proteolytic activity assessment use of the chromogenic substrate hide powder azure. Technologia, **7**(31), 40-46.

Carrie, M. S. (1952). The recovery of wool from skin pieces. Vol. 3. LASRA Fellmongers and Hide Processors annual conference New Zealand Leather and Shoe Research Association.

Carrie, M. S., Moore, J. B., & Chisholm, J. (1956). The depilation of sheep skins with acetic acid. New Zealand Journal of Science and Technology, **38**(B), 19-30.

Cay, M., Kielty, C., & Shuttleworth, A. (1997). Microfibrillar elements of the dermal matrix. Microscopy Research and Technique, **38**(4), 413-427.

Challenge tanning machinery. (1977). USA: Challenge-Cook Bros,. INC.

Charonis, A. S., & Tsilibary, E. C. (1990). Assembly of basement membrane proteins. M. W. Adair, & R. P. Mecham (Editors), Organisation and assembly of plant and animal extracellular matrix (pp. 85-117). San Diego: Academic Press.

Chen, W., Zhang, Y., Qi, J., Tian, J., Jiang, Y., & Yan, X. (2000). Research about new oxidative unhairing method using chlorine dioxide solution. Journal of the American Leather Chemists Association., **95**(5), 272.

Cheng, S., Hu, H., Shen, S., Takagi, H., Asano, M., & Tsai, Y. (1995). Production and characterisation of keratinase of a feather-degrading *Bacillus licheniformis* PWD-1. Bioscience, Biotechnology, and Biochemistry., **59**(12), 2239-2243.

Chiquet-Ehrissmann, R. (1999). Part 3. Tenascins. T. Kreis, & R. Vale (Editors), Guidebook to the extracellular matrix, anchor, and adhesion proteins. Oxford: Oxford University Press.

Chothia, C., & Jones, E. Y. (1997). The molecular structure of cell adhesion molecules. Annual Reviews of Biochemistry, **66**, 823-862.

Christner, J. (1988). The pros and cons of a hair save process in the beamhouse. Journal of the American Leather Chemists Association., **83**(6), 183-192.

Chu, M., Pan, T., Conway, D., Saitta, B., Stokes, D., Kuo, H., Glanville, R. W., Timpl, R., Man, K., & Deutzmann, R. (1990). The structure of type VI collagen. R. Fleischmajer, B. R. Olsen, & K. Kühn (Editors), Structure, molecular biology, and pathology of collagen (Vol. 580). New York : New York Academy of Sciences.

Clegg, J. E. (Inventor) (1989). Complete specification "Removing fibres from skin". (NZ Patent 2306760).

Cooper, S. M. (1997). (Report No. RR102). Palmerston North: New Zealand Leather and Shoe Research Association.

Cooper, S. M. (1998a). Elastin and processing. LASRA Report of the annual conference for fellmongers and Hide processors and tanners and leather technologists Palmerston North: New Zealand Leather and Shoe Research Association (Inc).

Cooper, S. M. (1998b). (Report No. RR118). Palmerston North: New Zealand Leather and Shoe Research Association.

Cooper, S. M., & DasGupta, S. (2002). (Report No. RR29). Palmerston North: New Zealand Leather and Shoe Research Association.

Cooper, S. M., & Lowe, E. K. (1997). (Report No. RR104). Palmerston North: New Zealand Leather and Shoe Research Association.

Cooper, S. M., & Lowe, E. K. (2000). (Report No. RR136). Palmerston North: New Zealand Leather and Shoe Research Association.

Couchman, J. R., McCarthy, K. J., & Woods, A. (1991). Proteoglycans and glycoproteins in hair follicle development and cycling. K. S. Stenn, A. G. Messenger, & H. P. Baden (Editors), The molecular and structural biology of hair (Vol. 642). The New York Academy of Sciences.

Coulson, R. G. (1969). The SATRA Break/Pipiness Scale. Journal of the American Leather Chemists Association., **64**, 648.

Cubis, G. (1977). Eyes on chemical shearing... will it provide the answer to rising costs? New Zealand Journal of Agriculture, **134**(1), 37.

D'Arcy, J. B. (1979). Sheep management and wool technology. Brisbane: Watson

Ferguson.

Daniels, R. (2002a). Back to basics part 1 of 9: Raw material and preservation. World Leather, **15**(2), 52-53.

Daniels, R. (2002b). Back to basics part 2 of 9: Preparation for tanning (i). World Leather, **15**(3), 56.

Dayanandan, A., Kanagaraj, J., Sounderraj, L., Govindaraju, R., & Suseela Rajkumar, G. (2003). Application of an alkaline protease in leather processing: An ecofriendly approach. Journal of Cleaner Production, **11**(6), 533-536.

Deb Choudhury, S., Allsop, T., Passman, A., & Norris, G. E. (2006). Use of a proteomics approach to identify favourable conditions for production of good quality lambskin leather. Analytical and Bioanalytical Chemistry, **384**, 723-735.

Dempsey, M. (1976). Some natural and other defects in pelt and leather. Fellmongers and hide processors annual conference New Zealand Leather and Shoe Research Association.

Dempsey, M. (1984). Hide, Skin and Leather Defects: A Guide to their Microscopy. Palmerston North, New Zealand: New Zealand Leather and Shoe Association.

Ding, H., Sun, L., & Liang, C. (2005). Action of pancreatin on residual epidermis of goatskin - examination via histological changes during bating. Journal of the Society of Leather Technologists and Chemists., **89**(4), 141-144.

Dingle, J. T. (1976). The role of neutral and acid proteinases in connective tissue turnover. D. W. Ribbons, & K. Brew (Editors), Proteolysis and physiological regulation. New York: Academic Press.

Edmonds, R. L. (2001). Replacement of sulfide with auxiliary chemicals. Report of the fifty second annual LASRA Conference Palmerston North: New Zealand Leather and Shoe Research Association.

Edmonds, R. L. (2002). The effect of elastin on sheep pelt area yields. Report of the fifty third annual LASRA conference Palmerston North: New Zealand Leather and Shoe Research Association.

Edmonds, R. L. (2003a). Tanners Technical Advisory Committee. (Report No. IR119). Palmerston North: New Zealand Leather and Shoe Research Association.

Edmonds, R. L. (2003b). Investigation into prefleshing and dewatering. Report of the fifty fourth Annual LASRA conference Palmerston North: New Zealand Leather and Shoe Research Association.

Edmonds, R. L. (2007). Depilation accelerants. Report of the 58th Annual LASRA conference Palmerston North: New Zealand Leather and Shoe Research Association.

Edmonds, R. L., Deb Choudhury, S., Haverkamp, R. G., Birtles, M., Allsop, T. F. &. Norris, G. E. (2008). Using proteomics, immunohistology, and atomic force microscopy to characterize surface damage to lambskins observed after enzymatic dewooling. Journal of Agricultural and Food Chemistry, **56**(17), 7934-7941.

El Baba, H. A. M., Covington, A. D., & Davighi, D. (1999). The effects of hair shaving on unhairing reactions: Part 1 The environmental impact. Journal of the Society of Leather Technologists and Chemists., **84**(4), 200-203.

El Baba, H. A. M., Covington, A. D., & Davighi, D. (2000). The effects of hair shaving on unhairing reactions Part 2: A new mechanism of unhairing. Journal of the Society of Leather Technologists and Chemists., **84**(1), 48-53.

Engel, J., & Bozic, D. (1999). Part 3. Modules in ECM and adhesion molecules. T. Kreis, & R. Vale (Editors), Guidebook to the Extracellular matrix, anchor, and adhesion proteins. Oxford: Oxford University Press.

Engvall, E., Hessle, H., & Klier, G. (1986). Molecular assembly, secretion, and matrix deposition of type VI collagen. Journal of Cell Biology., **102**, 703-710.

Eskolin, M., Marjoniemi, M., & Mantysalo, E. (1993). Anisotropy of physical characteristic functions of sheep leather.; Production of hides, skins, wool and hair. Proceedings of the joint EAAP-CIHEAM-EC-CUZF-MAFRA Symposium Tampere, Finland: Tampere University of Technology, Laboratory of Fur and Leather Technology.

Fadal, A. A., & Speranza, M. (1996). The hair recovery liming system. Leather Manufacturer, **113**(9), 11-16.

Faust, P. L., Kornfeild. S., & Chirgwin, J. M. (1985). Proceedings of the National Acadamy of Sciences, **82**, 4910-4914.

Felicjaniak, B. (1985). Studies in enzymatic unhairing: Part I. The influence of pigskin alkalisation on destruction of skin grain during depilation with pancreatic enzymes. Journal of the Society of Leather Technologists and Chemists., **69**(6), 160-163.

Fisher, L. W. (1999a). Part 3. Biglycan (BGN). T. Kreis, & R. Vale (Editors), Guidebook to the extracellular matrix, anchor, and adhesion proteins. Oxford: Oxford University Press.

Fisher, L. W. (1999b). Part 3. Decorin. T. Kreis, & R. Vale (Editors), Guidebook to the extracellular matrix, anchor, and adhesion proteins. Oxford: Oxford University Press.

Fisher, L. W., Heegaard, A., Vetter, U., Vogel, W., Just, W., Termine, J. D., & Young, M. F. (1991). Human bigycan gene: Putative promoter, intron-exon junctions, and chromosomal localization. Journal of Biological Chemistry, **266**(22), 14371-14377.

Fleischmajer, R., Pelish, J. S., Burgeson, R. E., Shaikh-bahai, F., & Timpl, R. (1990). Type I and type III collagen interactions during fibrillogenesis. R. Fleischmajer, B. R. Olsen, & K. Kühn (Editors), Structure, molecular biology, and pathology of collagen Vol. **580**. New York: New York Academy of Sciences.

Foroughi, F., Keshavarz, T., & Evans, C. S. (2006). Specificities of proteases for use in leather manufacture. Journal of Chemistry, Technology, and Biotechology., **81**, 257-261.

Fukunaga, S., Himoto, A., Yamakawa, I., Takenouchi, K., & Nakamura, F. (2006). Morphological characteristics of Yeso sika deer skin in comparison with bovine and porcine skins. Hikaku Kagaku, **52**(2), 33-46.

Funderburg, J. L., & Conrad, G. W. (1989). Detection and purification of corneal keratan sulfate proteoglycan from non corneal tissues. H. Greiling, & J. E. Scott (Editors), Keratan sulfate chemistry, biology, chemical pathology. London: The Biochemical Society.

Galarza, B. C., Goya, L. M., Cantera, C. S., Garro, M. L., Reinos, H. E., & López, M. I. (2004). Fungal biotransformation of bovine hair Part 1: Isolation of fungus with

keratinolytic activity. Partial characterization of crude fungal extracts. Journal of the Society of Leather Technologists and Chemists., **88**, 93-98.

GE Healthcare UK Limited. (2006). Amersham ECL Western blotting detection reagents and analysis system. Product booklet RPN2106. Buckinghamshire, UK: General Electric Company.

Gehring, A. G., DiMaio, G. L., Marmer, W. N., & Mazenko, C. E. (2002). Unhairing with proteolytic enzymes derived from *Streptomyces griseus*. Journal of the American Leather Chemists Association., **97**(10), 406-411.

Germann, H.-P. (1997). The evolution of the unhairing process as influenced by technological, economic and ecological considerations. Journal of the American Leather Chemists Association., **92**(4), 84-92.

Gibb, A. (1987). Wool recovery from band knife shearing. Report of the 38th annual LASRA Fellmongers and Hide Processors Annual Conference Palmerston North: New Zealand Leather and shoe research association.

Glanville, R. W. (1987). Type IV Collagen. R. Mayne, & R. E. Burgeson (Editors), Biology of extracellular matrix: A series - Structure and function of collagen types. Florida: Academic Press.

Glover, J. C., Hughes, I. R., & Vivian, G. W. (1975). Mould growth on pickled pelt. Journal of the Society of Leather Technologists and Chemists., **59**, 74-78.

Gordon, A. J. (1980). The measurement of, and factors affecting, the strength of attachment of wool to the skin of sheep. Australian Journal of Experimental, Agriculture, and Animal Husbandry., **20**, 40-49.

Gordon, M. K., Gerecke, D. R., Dublet, B., Van der rest, M., Sugrue, S. P., & Olsen, B. R. (1990). The structure of type XII Collagen. R. Fleischmajer, B. R. Olsen, & K. Kühn (Editors), Structure, molecular biology, and pathology of collagen (Vol. 580pp. 8-16). New York: New York Academy of Sciences.

Gray, H. (1918). Anatomy of the human body. Philadelphia : Lea & Febiger.

Green, G. H. (1952). Unhairing by means of enzymes (A review of literature). Journal

of the Society of Leather Technologists and Chemists., **36**, 127-134.

Grinnell, F. (1982). Cell-Collagen interactions: Overview. Methods in Enzymology., **82**, 499-503.

Guns, J., Lacroix, J., & Buwalda, P. L. (Inventors) (2003). Depilatory paint thickener comprising an anylopectin [sic] starch. (US 6589293).

Haines, B. M. (1981). Leather under the microscope. Northampton: British Leather Manufacturers' Research Association.

Haines, B. M. (1984a). Quality Rawstock. Journal of the American Leather Chemists Association., **79**, 329.

Haines, B. M. (1984b). Twentieth Procter memorial lecture: The skin before tannage - Procter's view and now. Journal of the Society of Leather Technologists and Chemists., **68**, 57-70.

Haines, B. M. (2006). The fibre structure of leather. M. Kite., & R. Thomson. (Editors), Conservation of leather and related materials. Oxford: Elsevier.

Hall, D. E. (1999). Part 3. Vitronectin. T. Kreis, & R. Vale (Editors), Guidebook to the extracellular matrix, anchor, and adhesion proteins. Oxford: Oxford University Press.

Harris, D. (1974). Dictionary of the terms used in the hides, skins, and leather trade. Washington D.C.: US department of Agriculture.

Harris, D. C. (1998). Nonlinear Least-Squares Curve Fitting with Microsoft Excel Solver. Journal of Chemical Education., **75**(1), 119-121.

Hawkins, S. C., & Wassenberg, J. (1997). Endogenous enzyme fellmongering - skin structure and properties. 24th IULTCS Congress proceedings (p. 511).

Hay, E. D. (1964). Secretion of a connective tissue protein by developing epidermis. W. Montagna, & W. C. Lobitz Jr. (Editors), The epidermis. London: Academic Press.

He, Q., Yao, K., Sun, D., & Shi, B. (2006). Biodegradability of wastewater from enzymatic soaking and unhairing processes in leather manufacture. Journal of the American Leather Chemists Association., **101**, 217-222.

Heller-Harrison, R. A., & Carter, W. G. (1984). Pepsin generated type VI collagen is a degradation product of GP140. Journal of Biological Chemistry, **259**(11), 6858-6864.

Hukins, D. W. L. (1984). Tissue components. D. W. L. Hukins (Editor), Connective tissue matrix. London: Macmillan Press.

Hynes, R. (1999). Part 3. Fibronectins. T. Kreis, & R. Vale (Editors), Guidebook to the extracellular matrix, anchor, and adhesion proteins. Oxford: Oxford University Press.

IHCworld. (2007) Citrate buffer antigen retrieval protocol [Web Page]. URL http://www.ihcworld.com/_protocols/epitope_retrieval/citrate_buffer.htm [2007].

IUP 2. (2000). Sampling. Journal of the Society of Leather Technologists and Chemists., **84**, 303.

IUP 36. (2000). Measurement of leather softness. Journal of the Society of Leather Technologists and Chemists., **84**, 377.

IUP 6. (2000). Measurement of tensile strength and percentage elongation. Journal of the Society of Leather Technologists and Chemists., **84**, 317.

IUP 8. (2000). Measurement of tear load-double edge tear. Journal of the Society of Leather Technologists and Chemists., **84**, 327.

Jackways, G. D. (1987). (Report No. RR38). Palmerston North: New Zealand Leather and Shoe Research Association.

James, S. J., & James, C. (2002). Meat Refrigeration. Cambridge: Woodhead Publishing.

Jarratt, P. (1999). Bioclip: A different approach to wool recovery. Report of the fiftieth Annual Conference for Fellmongers and Hide Processors and Tanners and Leather Technologists New Zealand Leather and Shoe Research Association.

Jian, S., Wenyi, T., & Wuyong, C. (2007). Mathematical analysis of depilation using combinations of amylase and protease. Journal of the Society of Leather Technologists and Chemists., **91**(5), 212-215.

Jiang, L., He, L., & Fountoulakis, M. (2004). Comparison of protein precipitation

methods for sample preparation prior to proteomic analysis. Journal of Chromatography A, **1023**(2), 317-320.

Jin, B., Yang, Y., & Chen, L. (1996). The study on the histochemistry of enzyme depilation mechanism. The 3rd Asian international conference of leather science and technology.

Kaminska, R., Helisalmi, P., Harvima, R. J., Naukkarin, A., Horsmanheimo, M., & Harvima, I. T. (1999). Focal dermal-epidermal separation and fibronectin cleavage in basement membrane by human mast cell tryptase. Journal of Investigative Dermatology., **113**(4), 567-573.

Katunuma, N., Umezawa, H., & Holzer, H. (1983). Proteinase inhibitors - Medical and biological aspects. Berlin: Springer-Verlag.

Keene, D. R., Engvall, E., & Glanville, R. W. (1988). Ultrastructure of type VI collagen in human skin and cartilage suggests an anchoring function for this filamentous network. Journal of Cell Biology., **107**, 1995-2006.

Keene, D. R., Ridgway, C. C., & Iozzo, R. V. (1998). Type VI Microfilaments Interact with a Specific Region of Banded Collagen Fibrils in Skin. J. Histochem. Cytochem., **46**, 215-220.

Keller, V. W., & Heidemann, E. (1989). The quantitative composition of the skin papillary layer and its transform in the beamhouse processes. Das Leder, **40**(7), 140-149.

Keller, W. (1990). The quantitative composition of the skin papillary layer and its transformation in the beamhouse processes. Leder Und Hautmarkt, Gerbereiwissenschaft Und Praxis, **42**, 117-127.

Kemp, P. D. (1983). Fine structures of the grain surface and factors influencing loss of 'enamel'. BLMRA Journal, **26**(11), 151-153.

Kleinman., H. K. (1982). Fibroblast adhesion to collagen substrate. Methods in Enzymology., **82**, 503-508.

Knaflic, F., & Miller, F. (Inventors) (1980). Lime-free and sulfide-free liming process.

(US patent 4229175).

Kornfeld, R., & Kornfeld, S. (1980). Structure of glycoproteins and their oligosaccharide units. W. J. Lennarz (Editor), The biochemistry of glycoproteins and proteoglycans (pp. 1-34). New York: Plenum Press.

Kozlowski, G. P., & Calhoun, M. L. (1969). Microscopic anatomy of the integument of sheep. Americal Journal of Veterinary Research., **30**(7), 1267-1279.

Kraut, J. (1977). Serine proteases: Structure and mechanism of catalysis. Annual Review of Biochemistry., **46**, 331-58.

Kritzinger, C. C. (1948). Studies on globular proteins of animal skin. Journal of the American Leather Chemists Association., **33**, 675-689.

Kronick, P. L., & Iandola, S. (1997). Persistence of minority macromolecules of hide through the beamhouse. II. Removal of collagen type XIV. Journal of the American Leather Chemists Association., **92**, 172-178.

Kronick, P. L., & Iandola, S. (1998). Persistence of minority macromolecules of hide through the beamhouse. III. Persistence of decorin. Journal of the American Leather Chemists Association., **93**, 148-155.

Kronick, P. L., Maleeff, B. E., & Dahms, M. P. (1991). Removal of collagens VI and XII by beamhouse chemistry. Journal of the American Leather Chemists Association., **86**(6), 209-224.

Kucharz, E. J. (1992). The collagens: Biochemistry and pathophysiology. Berlin: Springer-Verlag.

Kühn, K. (1987). The classical collagens: Types I, II, and III. R. Mayne, & R. E. Burgeson (Editors), Biology of extracellular matrix: A series - Structure and function of collagen types (pp. 1-37). Florida: Academic Press.

Kuo, H. J., Maslen, C. L., Keene, D. R., & Glanville, R. W. (1997). Type VI collagen anchors endothelial basement membranes by interacting with type IV collagen. Journal of Biological Chemistry, **272**, 26522-26529.

Lander, A. D. (1999). Proteoglycans. T. Kreis, & R. Vale (Editors), Guidebook to the

extracellular matrix, anchor, and adhesion proteins. Oxford: Oxford University Press.

Lange, S., Laing, R. M., Niven, B. E., Scobie, D. R., Young, S. R., & Gore, S. E. (2002). Deer leather as a material for outerwear. Journal of the Society of Leather Technologists and Chemists., **86**(4), 157-162.

Leprince, P., Rogister, B., Delrée, P., Lefebvr, P. P., Rigo, J., & Moonen, G. (1990). Multiple roles for plasminogen activator system in nervous system development. B. W. Festoff (Editor), Serine proteases and their serpin inhibitors in the nervous system. New York: Plenum Press.

Letchworth, D., & Colton, D. A. (Inventors) (1979). Electronically controlled apparatus for electrolytic depilation. (US Patent 4,155,363).

Levenspiel, O. (1999). Chemical reaction engineering. New York: John Wiley & Sons.

Liang-jun, Y. (1987). A brief technological process of making pigskin full grain shoe upper leather. Vol. 19. IULTCS Congress proceedings.

Limat, A., Breitkreutz, D., Stark, H., Hunziker, T., Thikoetter, G., Noser, F., & Fusenig, N. E. (1991). Experimental modulation of the differentiated phenotype of keratinocytes from epidermis and hair follicle outer root sheath and matrix. K. S. Stenn, A. G. Messenger, & H. P. Baden (Editors), The molecular and structural biology of hair (Vol. 642pp. 125-147). The New York Academy of Sciences.

Lowe, E. K. (1996). Electrolytically enhanced depilation. Vol. 47. LASRA Fellmongers and Hide Processors Annual Conference Palmerston North.

Lowe, E. K. (1997). How CO2 Deliming Influences Bate performance and Leather quality. Report of the forty-eighth Annual conference of Fellmongers and Hide Processors Palmerston North: New Zealand Leather and Shoe Research Association (Inc).

Lowe, E. K. (1999). (Report No. RR120). Palmerston North: New Zealand Leather and Shoe Research Association.

Lowe, E. K. (2000). (Report No. RR132). Palmerston North: New Zealand Leather and Shoe Research Association.

Lowe, E. K., & Cooper, S. M. (1998). (Report No. RR106). Palmerston North: New Zealand Leather and Shoe Research Association.

Lowe, E., Cooper, SM., & Passman, A. (2000). Measurement of elastin content in skin tissue and process liquors. Journal of the Society of Leather Technologists and Chemists., **84**, 227-230.

Ludvik, J. (2000). The Scope for Decreasing Pollution Load in Leather Processing. (Report No. US/RAS/92/120/11-51). www.intracen.org/leatherline: UNIDO.

Macedo, A. J., Beys de Silva, W. O., Gava, R., Driemeier, D., Heriques, J. A. P., & Termignomi, C. (2005). Novel Keratinase from *Bacillus subtilis* S14 exhibiting remarkable dehairing capabilities. Applied Environmental Microbiology., **71**(1), 594-596.

Maclaren, J. A., & Milligan, B. (1981). Wool science The chemical reactivity of the wool fibre. Australia: Science Press.

Maddocks, I. G., & Jackson, N. (1988). Structural studies of sheep, cattle & goat skin. Blacktown NSW Australia: C.S.I.R.O.

Marmer, W. N., & Dudley, R. L. (2005a). Oxidative dehairing by sodium percarbonate. Journal of the American Leather Chemists Association., **100**, 427-431.

Marmer, W. N., & Dudley, R. L. (2005b). Rapid oxidative dehairing using alkaline hydrogen peroxide and potassium cyanate. Journal of the American Leather Chemists Association., **100**(5), 165-173.

Marmer, W. N., & Dudley, R. L. (2006). The oxidative degradation of keratin (wool and bovine hair). Journal of the American Leather Chemists Association., **101**.

Marmer, W. N., & Dudley, R. L. (2007). Oxidative dehairing: to relime or not to relime. XXIX IULTCS Congress (p. #25).

Mason, I. G. (1987). H2S: risks in pelt processing. Report of the 38th annual conference of fellmongers and hide processors Palmerston North: New Zealand Leather and Shoe Research Association.

MATLAB, (2004). The Mathworks Inc., (Version 7.0.0.19920(R14))

Matoltsy, A. G. (1965). Soluble prekeratin. A. G. Lyne, & B. F. Short (Editors), Biology of the skin and hair growth. Sydney: Angus and Robertson.

Maxwell, M. E. (1945). Bacteria responsible for the loosening of wool on sheepskins. F. G. Lennox., M. E. Maxwell., & W. J. Ellis.Fellmongering investigations papers I to XII Vol. 184 (pp. 45-55). Commonwealth of Australia Council for Scientific and Industrial Research.

Mecham, R. P. (1999). Part 3. Elastin. T. Kreis, & R. Vale (Editors), Guidebook to the Extracellular matrix, anchor, and adhesion proteins. Oxford: Oxford University Press.

Mier, P. D., & Cotton, D. W. K. (1976). The molecular biology of skin. Oxford: Blackwell Scientific Publications.

Money, C. A. (1996). Unhairing and dewooling - Requirements for quality and the environment. Journal of the Society of Leather Technologists and Chemists., **80**(175), 175-186.

Montagna, W., & Parakkal, P. F. (1974). The structure and function of skin. New York: Academic Press.

Mozersky, S. M., & Bailey, D. G. (1992). Hide powder azure and azocoll as substrates for assay of the proteolytic activity of bate. Journal of the American Leather Chemists Association., **87**(8), 287-295.

Mozersky, S. M., Wildermuth, R., & Marmer, W. N. (2003). Estimation of the sulfated glycosaminoglycan content of bovine skin with Alcian blue. Journal of the American Leather Chemists Association., **98**(9), 337-343.

Mozersky, S. M., Wildermuth, R., & Marmer, W. N. (2005). The relative proteolytic activity of pancreatic bate in media of low and high salt content. Journal of the American Leather Chemists Association., **100**(10), 396-400.

Mujahid, S., Pachan, T., & Wang, C. (2007). Improved solublization of surface proteins from *Listeria monocytogenes* for 2-DE. Electrophoresis, **28**, 3998-4007.

Myers, E. R., Armstrong, C. G., & Mow, V. C. (1984). Swelling pressure and collagen tension. D. W. L. Hukins (Editor), Connective tissue matrix. London: Macmillan Press.

Nagorcka, B. N., Dollin, A. R., Hollis, D. E., & Beaton, C. D. (1995). A technique to quantify and characterize the density of fibres and follicles in the skin of sheep. Australian Journal of Agricultural Research, **46**, 1525-1534.

Nakanishi, T., & Yamamoto, T. (1974). Action and specificity of a *Streptomyces* alkalophilic proteinase. Agricultural and Biological Chemistry, **38**(12), 2391-2397.

Neame, P. J., Choi, H. U., & Rosenberg, L. C. (1989). The primary structure of the core protein of the small. leucine-rich proteoglycan (PG I) from bovine articular cartilage. Journal of Biological Chemistry, **264**(15), 8653-8661.

Negri, A., Rankin, D. A., Nelson, W. G., & Rivett, D. E. (1996). A transmission electron microscope study of covalently bound fatty acids in the cell membranes of wool fibres. Textile Research Journal., **66**(8), 491-495.

North, M. J. (1989). Prevention of unwanted proteolysis. R. J. Beynon, & J. S. Bond (Editors), Proteolytic enzymes a practical approach. Oxford: IRL Press at Oxford Press.

O'Flaherty, F., Roddy, W. T., & Lollar, R. M. (1956). The chemistry and technology of leather Volume 1: The preparation for tannage. New York: Reinhold Publishing Corporation.

Olsen, B. R., & Ninomiya, Y. (1999). Part 3. Collagens. T. Kreis, & R. Vale (Editors), Guidebook to the extracellular matrix, anchor, and adhesion proteins. Oxford: Oxford University Press.

Onifade, A. A., Al-Sane, N. A., & Al-Musallam, A. A. A.-Z. S. (1998). A review: Potentials for biotechnological applications of keratin-degrading micro-organisms and their enzymes for nutritional improvement of feathers and other keratins as livestock feed resources. Bioresourse Technology, **66**, 1-11.

Ornes, C. L., Roddy, W. T., & Mellon, E. F. (1960). Study of the effect of elastic tissue of hide on the physical properties of leather. Journal of the American Leather Chemists Association., **55**, 600-621.

Ornstein, L., & Davis, B. J. (1964). Disc electrophoresis I. Background and theory. Annals of the New York Acadamy of Sciences, **121**, 321-349.

Orwin, D. F. G. (1979). The cytology and cytochemistry of the wool follicle. The International Review of Cytology, **60**, 331-375.

Passman, A. (Director) New Zealand: New Zealand Leather and Shoe Research Association. Personal communication.

Passman, A. (1984). Prefleshing for fellmongery processing. LASRA Fellmongers and Hide Processors Annual Conference Palmerston North: NZ LASRA.

Passman, A. (1986). Non lime thickeners for depilatory paints. Report of the thirty-seventh annual conference of fellmongers and hide processors (pp. 43-54). New Zealand Leather and Shoe Research Association.

Passman, A. (1997). The prospects, advantages and risks of replacing sulfide depilation technology with enzyme. LASRA Fellmongers and Hide Processors Annual Conference, **48**, 13-22.

Passman, A. (2002). Conference Opening. Report of the fifty third Annual LASRA conference Palmerston North: New Zealand Leather and Shoe Research Association (Inc).

Pauckner, W., & Herfeld, H. (1968). Investigations on the drying process for leather, IV The effect of different fatliquoring materials on drying time, fat distribution, visible structure and physical properties of leather in vacuum drying. Das Leder, **19**, 101-107.

Paul, R. G., Mohamed, I., Davighi, D., Covington, A. D., & Addy, V. L. (2001). The use of neutral protease in enzymatic unhairing. Journal of the American Leather Chemists Association., **96**(5), 180-185.

Pelczar, M. J. Jr., Chan, E. C. S., & Krieg, N. R. (1993). Microbiology: Concepts and applications. New York: McGraw-Hill.

Peterkofsky, B. (1982). Bacterial Collagenase. Methods in Enzymology., **82**, 453471.

Phillips, D. (Research scientist) New Zealand: Ag research. Personal communication.

Pitts, J. E., Crawford, M. D., Nugent, P. G., Wester, R. T., Cooper, J. B., Mantyla, A., Fagestrome, R., & Nevalain, H. (1995). The three dimensional x-ray crystal structure of the aspartic proteinase native to *Trichoderma Reesei* complexed with a rennin inhibitor

CP-80794. K. Takahashi (Editor), Aspartic proteinase: Structure, function, biology, and biomedical implications. New York: Plenum Press.

Redwood, M. (1975). The development, use and relevance of concrete mixers or hide processors in the modern tannery. Journal of the Society of Leather Technologists and Chemists., **59**(1).

Reich, G., Bradt, J., Mertig, M., Pompe, W., & Taeger, (1997). T. Scanning probe microscopy - An useful tool in leather research. 24th IULTCS congress proceedings.

Reichardt, L. F. (1999). Part 3. Introduction. T. Kreis, & R. Vale (Editors), Guidebook to the extracellular matrix, anchor, and adhesion proteins. Oxford: Oxford University Press.

Ricard-Blum, S., Dublet, B., & van der Rest, M. (2000). Unconventional Collagens: Types VI, VII, VIII, IX, X, XII, XIV, XVI and XIX. Oxford: Oxford University Press.

Robbins, T. L., & Manley, R. (2002). Odor prevention and control in process plants. Chemical Engineering, **109**(2), 50-55

Roberts, G. P., & Brunt, J. (1985). Identification of an epidermal cell-adhesion glycoprotein. Biochemical Journal, **232**, 67-70.

Rodén, L. (1980). Structure and metabolism of connective tissue proteoglycans. W. J. Lennarz (Editor), The biochemistry of glycoproteins and proteoglycans. London: Plenum Press.

Roether, E. (1976). Leather technical glossary in six languages. Darmstadt: Roetherprint.

Rollins, B. J., Cathcart, M. K., & Culp L.A. (1982). Glycoconjugates in cellular adhesion and aggregation. Section 1: fibronectin-proteoglycan binding as the molecular basis for fibroblast adhesion to extracellular matrices. Horowitz (Editor), The glycoconjugates. Volume III: Glycoproteins, glycolipids, and proteoglycans Part A. New York: Academic Press.

Rose, C., Suguna, L., Rajini, R., Samivelu, N., Rathinasamy, V., Ramalingam, S., Iyappan, K., Parvathaleswara, T., & Ramasami, T. (Inventors) (2007). Process for lime

and sulfide free unhairing of skins or hides using animal and/or plant enzymes. (US Patent 7198647B2).

Rosenbusch, K., & Kliegl, W. (1971). New aspects of the clarification of tannery waste waters from oxidative unhairing. Journal of the American Leather Chemists Association., **66**(7), 146.

Saksela, O., Alitalo, K., Kiistala, U., & Vaheri, A. (1981). Basal lamina components in experimentally induced skin blisters. Journal of Investigative Dermatology., **77**(3), 283-286.

Salvesen, G., & Nagase, H. (1989). Inhibition of proteolytic enzymes. R. J. Beynon, & J. S. Bond (Editors), Proteolytic enzymes a practical approach. Oxford: IRL Press at Oxford University Press.

Saravanabhavan, P., Thanikaivelan, P., Rao, J. R., & Nair, B. U. (2005). An enzymatic beamhouse process coupled with semi-metal tanning and eco-benign post tanning leads to cleaner leather production. Journal of the American Leather Chemists Association., **100**(5), 174-186.

Sasaki, T., & Timpl, R. (1999). Part 3. Laminins. T. Kreis, & R. Vale (Editors), Guidebook to the extracellular matrix, anchor, and adhesion proteins. Oxford: Oxford University Press.

Sawaya, M. E. (1991). Steroid chemistry and hormone controls during the hair follicle cycle. Mollecular Structure, and Biology of Hair, **642**, 376-84.

Schraeder, C. E., Ervin, R. T., & Eberspacher, J. L. (1998). Economic analysis of the feasibility of using enzymes in the unhairing process. Journal of the American Leather Chemists Association., **93**(9), 265-271.

Scobie, D. R., Bray, A. R., & Merrick, N. C. (1998). Medullation and average fibre diameter vary independently in the wool of Romney sheep. New Zealand Journal of Agricultural Research, **41**, 101-110.

Scroggie, J. G. (1970). The interaction between "chlorine dioxide" and collagen. Journal of the American Leather Chemists Association., **55**(5), 216-234.

Sharphouse, J. H. (1971). Leather technician's handbook. London: Vernon Lock.

Shevchenko, A., Wilm, M., Vorm, O., & Mann, M. (1996). Mass spectrometric sequencing of protein from silver-stained polyacrylamide gels. Analytical Chemistry, **68**, 850-858.

Shi., B., Lu., X., & Sun., D. (2003). Further investigations of oxidative unhairing using hydrogen peroxide. Journal of the American Leather Chemists Association., **98**(5), 185-192.

Shivas, S. A. (1995). H_2S in the Tannery. Leather Manufacturer, **113**(8).

Sigmaaldrich. (2006) Matrix_Metalloproteinases [Web Page]. URL http://www.sigmaaldrich.com/Area_of_interest/Biochemicals/Explorer/Cell_Signaling_Enzymes/Matrix_Metalloproteinases.html [1916, December 6].

Simoncini, A. (1987). Biotechnology in the tanning industry. Journal of the American Leather Chemists Association., **82**(8), 226-241.

Simpson, D. M., Beyon, R. J., Robertson, D. H. L., Loughran, M. J., & Haywood, S. (2004). Copper associated liver disease: A proteomics study of copper challenge in a sheep model. Proteomics, **4**, 524-536.

Sivasubramanian, S., Murali Manohar, B., & Puvanakrishnan, R. (2008). Mechanism of enzymatic dehairing of skins using a bacterial alkaline protease. Chemosphere, **70**, 1025-1034.

Smidek, J., & Heidemann, E. (1987). Development of sulfide free and low grade sulfide depilation. Das Leder, **38**(3), 48-57.

SLC 7; IUC 10; BS 1309:7; Determination of nitrogen and hide substance. (1996). Society of leather chemists and technologists Official methods of analysis. The society of leather technologists and chemists.

SLC 4 ; IUC 4; Determination of substances (Fats and other solubles) soluble in dichloromethane. (1996). Society of leather technologists and chemists Official methods of analysis. The society of leather technologists and chemists.

Stanley, A. (1994). Cleaner contribution of enzymes. Leather, **196**(4642), 43-44,46,48.

Stanley, J. R., Woodley, D. T., Katz, S. I., & Martin, G. R. (1982). Structure and function of basement membrane. Journal of Investigative Dermatology., **79**, 69-72.

Stephens, L. J., Werkmeister, J. A., & Ramshaw, J. A. M. (1991). Use of immunohistology to study the connective tissue components of hides and skins. 21st IULTCS congress Spain.

Sternlicht, M. B., & Werb, Z. (1999). ECM proteinases. T. Kreis, & R. Vale (Editors), Guidebook to the Extracellular matrix, anchor, and adhesion proteins (Second edition ed., p. 505). Oxford: Oxford University Press.

Stewart, B. W. (Inventor) (2003). Method of hair removal by trans cutaneous application of laser light. (US Patent 6544255 B2).

Stewart, K. J. (1995). A quantitative ultrastructural study of collagen fibrils in human skin normal scars, and hypertrophic scars. Clinical Anatomy, **8**(5), 334-338.

Stirtz, T. (1965). Die Feinstruktur der Narbenmembran. Das Leder, **16**(8), 177.

Stirtz, T. (1982). Processes in the skin under the influence of bating enzymes Part 2. Changes in the hide substance through bating proteases with special emphasis on leather-forming collagen. Das Leder, **33**, 67-78.

Stocker, W., Grams, F., Baumann, U., Reinemer, P., Gomis-Ruth, F. X., McKay, D. B., & Bode, W. (1995). Protein Science, **4**, 823-840.

Straile, W. E. (1965). Root sheath-dermal papilla relationships and the control of hair growth. A. G. Lyne, & B. F. Short (Editors), Biology of the skin and hair growth. Sydney: Angus and Robertson.

Sundar, J. V., Balakrishnan, P. A., Chakrabarti, S., & Muralidharan, C. (2006). Sulfide free unhairing, Studies on ozone based depilation. Journal of the American Leather Chemists Association., **101**, 231-234.

Taña, J. C., & Taña, E. C. (Inventors) (1988). Procedure for removing the layer of hairy elements from a complete animal skin. (US Patent 4,770,010).

Tandt, H., Russell, A. E., Kohl, R. C., Rose, P. D., & Jackson-Moss, C. (1997). Inhibition of proteolytic activity causing grain enamel in leather. 24th IULTCS

Congress proceedings (p. 797).

Taylor, M. M., Bailey, D. G., & Feairheller, S. H. (1987). A review of the use of enzymes in the tannery. Journal of the American Leather Chemists Association., **82**(6), 153-165.

Taylor, M. M., Marmer, W. N., & Brown, E. M. (2006). Preparation and characterization of biopolymers derived from enzymatically modified gelatin and whey. Journal of the American Leather Chemists Association., **101**(6), 235-248.

Test and Research Institute for Shoe Manufacturing, P. (1976). About the fat content of leather. Schuh-Technik, **70**(8), 771-773.

Thanikaivelan, P. C. B., Bharath, C. K., Saravanabhavan, S., Anandhi, C., Rao, J. R., & Nair, B. U. (2006). Single step hair removal and fiber opening process simultaneous and successive addition of protease and a-amylase. Journal of the American Leather Chemists Association., **101**, 388-398.

Thanikaivelan, P., Bharath, C. K., Saravanabhavan, S., Rao, J. R., Chandrasekaran, B., Chandrababu, N. K., & Nair, B. U. (2007). Integrated hair removal and fibre opening process using mixed enzymes. Clean Technology and Environmental Policy, **9**, 61-68.

Thanikaivelan, P., Raghava Rao, J., & Unni Nair, B. (2000a). Development of a leather processing method on narrow pH profile: Part 1 standardisation of dehairing process. Journal of the Society of Leather Technologists and Chemists., **84**(6), 276-284.

Thanikaivelan, P., Raghava Rao, J., & Unni Nair, B. (2000b). Development of leather processing method in narrow pH profile: Part 2. Standardisation of tanning process. Journal of the Society of Leather Technologists and Chemists., **85**, 106.

Thanikaivelan, P., Rao, J. R., & Ramasami, T. (2005). Recent trends in leather making: processes, problems, and pathways. Critical Reviews in Science and Technology, 37-79.

The Mathworks inc. (2004). MATLAB (Version R14) : The Mathworks inc.

Timpl, R. (1999). Part 3. Nidogen. T. Kreis, & R. Vale (Editors), Guidebook to the Extracellular matrix, anchor, and adhesion proteins. Oxford: Oxford University Press.

Timpl, R., Aumailley, M., Gerl, M., Mann, K., Nurcombe, V., Edgar, D., & Deutzmann, R. (1990). Structure and function of laminin-Nidogen complex. R. Fleischmajer, B. R. Olsen, & K. Kühn (Editors), Structure, molecular biology, and pathology of collagen (Vol. 580). New York: New York Academy of Sciences.

Timpl, R., Wiedermann, H., van Delden, V., Furthmayer, H., & Kühn, K. (1981). A network model for the organisation of type IV collagen molecules in basement membranes. European Journal of Biochemistry, **120** , 203-211.

Toole, B. P. (1999). Part 3. Hyaluronan. T. Kreis, & R. Vale (Editors), Guidebook to the extracellular matrix, anchor, and adhesion proteins. Oxford: Oxford University Press.

Tschesche, H., & Macartney, H. W. (1983). Human Leucocyte Collagenase and regulation of activity. N. Katunuma, H. Umezawa, & H. Holzer (Editors), Proteinase inhibitors - Medical and biological aspects (pp. 211-228). Berlin: Springer-Verlag.

van der Loo, W. P. M., Gampert, A., Bottini, V., Ylla-Catala, J., Reaks, G. G., & Merget, E. (1999). International glossary of leather terms (2nd Edition). Northampton: International council of tanners.

van der Rest, M., & Mayne, R. (1987). Type IX Collagen. R. Mayne, & R. E. Burgeson (editors), Biology of extracellular matrix: A series - Structure and function of collagen types. Florida: Academic Press.

Veit, G., Hansen, U., Keene, D. R., Bruckner, P., Chiquet-Ehrismann, R., Chiquet, M., & Koch, M. (2006a). Collagen XII Interacts with Avian Tenascin-X through Its NC3 Domain. Journal of Biological Chemistry, **281**(37), 27461-27470.

Veit, G., Kobbe, B., Keene, D. R., Paulsson, M., Koch, M., & Wagener, R. (2006b). Collagen XXVIII, a novel von Willebrand Factor A domain-containing protein with many imperfections in the collagenous domain interacts with avian Tenascin-X through its NC3 domain. Journal of Biological Chemistry, **281**(6), 3494-3504.

Vivian, G. W. (1978). Hydrated lime as a depilatory paint thickener. LASRA Fellmongers and Hide Processors Annual Conference (pp. 46-58).

von Loutis, N., Stirtz, T., Fietzek, P. P., & Heidemann, E. (1984). Versuche zur chemischen Identifizierung von Typ IV Kollagen (Basalmembran-Kollagen) in der

Narbenschicht von Rindshäuten. Das Leder, **35**(5), 75-77.

Walsh, M. D., & Jass, J. R. (2000). Histologically based methods for detection of mucin. A. P. Corfield (Editor), Methods in molecular biology Volume 125: Glycoprotein methods and protocols. Totowa, New Jersey: Humana Press.

Wang, Y., Li, Z., Chen, M., Cheng, H., & Liao, L. (2005). Study on changes of collagen fibril structure in pigskin tissue after enzyme treatment. Journal of the Society of Leather Technologists and Chemists., **89**(2), 47-56.

Waters, P. J., & Price, S. M. (1989). Aqueous degreasing of Australian woolly sheepskins. XXth IULTCS congress texts of papers.

Webster, R. M., Walker, M. P., & Alexander, K. T. W. (1987). (Report No. LR-162). Northampton: British Leather Manufacturers Research Association.

Wegrowski, J., Bellon, G., & Rondoux, A. (1990). Human type VI collagen: Isolation and characterisation of α1 and α2 chains by two-dimensional preparative gel electrophoresis. *Biochemica et Biophysical Acta*, **1039**, 189-196.

Weiss, J. B. (1984). Collagens and collagenolytic enzymes. D. W. L. Hukins (Editor), Connective tissue matrix. London: Macmillan Press.

Wessel, D., & Flügge, U. I. (1984). A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. Analytical Biochemistry., **138**(1), 141-143.

Windus, W., & Showell, J. S. (1968). An interpretation of the mechanism of unhairing as a nucleophilic displacement. Journal of the American Leather Chemists Association., **63**, 258-274.

Woodley, D. T., Chang, C., Saadat, P., Ram, R., Liu, Z., & Chen, M. (2005). Evidence that anti-type VII collagen antibodies are pathogenic and responsible for the clinical, histological, and immunological features of epidermolysis bullosa acquisita. Journal of Investigative Dermatology., **124**(5), 958-964.

Yamato, K. (1995). Cathepsin E and Cathepsin D: Biosynthesis, processing and subcellular location. K. Takahashi Aspartic proteinase: Structure, function, biology, and

biomedical implications. Plenum Press.

Yates, J. R. (1964). Studies in depilation I. The effect of bacteriocides on the rate of wool loosening (sweating) of sheepskins. Journal of the American Leather Chemists Association., **59**(9), 650-662.

Yates, J. R. (1968a). Studies in depilation II. Structural changes in the wool follicle during bacterial wool loosening ("sweating"). Australian Journal of Biological Science, **21**, 361-374.

Yates, J. R. (1968b). Studies in depilation IV. Structural changes in the wool follicle during depilation with alkali and alkaline reducing systems. Australian Journal of Biological Science, **21**, 795-804.

Yates, J. R. (1968c). Studies in depilation V. Investigation of the relative depilatory efficiency of a number of commercially available enzyme systems. Journal of the American Leather Chemists Association., **63**(8), 464-473.

Yates, J. R. (1968d). Studies in depilation VI. Correlation of activity of depilatory enzyme systems with their activity against simple substrates. Journal of the American Leather Chemists Association., **63**(8), 474-479.

Yates, J. R. (1968e). Studies in depilation VII. Structural changes in the wool follicle during depilation with acid and enzyme systems. Australian Journal of Biological Science, **21**, 1249-1261.

Yates, J. R. (1969a). Studies in depilation IX. Effect of skin thickness and diffusion on the rate of depilation of sheepskins. Journal of the American Leather Chemists Association., **64**(2), 71-81.

Yates, J. R. (1969b). Studies in depilation VIII. A method for determining the depilatory activity of enzyme preparations on sheepskins using only small quantities of enzyme. Journal of the American Leather Chemists Association., **64**, 63-70.

Yates, J. R. (1972). Studies in depilation X. The mechanism of the enzyme depilation process. Journal of the Society of Leather Technologists and Chemists., **56**(5), 158-177.

Yongquan, L. (2001). Sheep-pelt bating with acid protease. Journal of the American

Leather Chemists Association., **96**(10), 398-400.

Yurchenco, P. D. (1990). Assembly of basement membranes. R. Fleischmajer, B. R. Olsen, & K. Kühn (Editors), Structure, molecular biology, and pathology of collagen Vol. **580**. New York: New York Academy of Sciences.

Zeichen, J., van Griensven, M., Albers, I., Lobenhoffer, P., & Bosch, U. (1999). Immunohistochemical localization of collagen VI in arthrofibrosis. Archives of Orthopaedic and Trauma Surgery, **119**(5-6).

Zimmermann, D. R. (1999). Part 3. Versican. T. Kreis, & R. Vale (Editors), Guidebook to the extracellular matrix, anchor, and adhesion proteins. Oxford: Oxford University Press.